REGULATION OF MALE GERM CELL APOPTOSIS

Roles of sex steroids and the cellular death receptors Fas and TNFR1

Virve Pentikäinen

Programme for Developmental and Reproductive Biology
Biomedicum Helsinki
University of Helsinki
Finland

and

Hospital for Children and Adolescents
Helsinki University Central Hospital
University of Helsinki
Finland

ACADEMIC DISSERTATION

Helsinki University Biomedical Dissertations No.13

To be publicly discussed with permission of
the Medical Faculty of the University of Helsinki,
in the Niilo Hallman Auditorium of the Hospital for Children and Adolescents,
on June 28th, 2002, at 12 noon.

Helsinki 2002

Supervisor

Docent Leo Dunkel, M.D.
Hospital for Children and Adolescents
Helsinki University Central Hospital
and
Programme for Developmental and Reproductive Biology
Biomedicum Helsinki
University of Helsinki
Helsinki, Finland

Reviewers

Professor Ismo Virtanen, M.D.
Department of Anatomy
University of Helsinki,
Helsinki, Finland

and

Professor John Eriksson, Ph.D.
Department of Biology
University of Turku
Turku, Finland

Official opponent

Professor Veli-Pekka Lehto, M.D.
Department of Pathology
University of Helsinki
Helsinki, Finland

ISBN 952-10-0605-6 (nid.)
ISBN 952-10-0606-4 (pdf)
ISSN 1457-8433
Yliopistopaino
2002

To Markku and Juho

Contents

Original Publications

This thesis is based on the following original publications, which are referred in the text by roman numerals.

I. Pentikäinen V, Erkkilä K, Suomalainen L, Parvinen M, Dunkel L: Estradiol acts as a germ cell survival factor in the human testis *in vitro*. *J Clin Endocrinol Metab* 85:2057-2067, 2000.

II. Pentikäinen V, Erkkilä K, Dunkel L: Fas regulates germ cell apoptosis in the human testis *in vitro*. *Am J Physiol* 276 (*Endocrinol Metab* 39): E310-E316, 1999.

III. Pentikäinen V, Erkkilä K, Suomalainen L, Otala M, Pentikäinen MO, Parvinen M, Dunkel L: TNFα downregulates the Fas ligand and inhibits apoptosis in the human testis. *J Clin Endocrinol Metab* 86: 4480-4488, 2001.

IV. Pentikäinen V, Suomalainen L, Erkkilä K, Martelin E, Parvinen M, Pentikäinen MO, Dunkel L: NF-κB activation in human testicular apoptosis. *Am J Pathol* 160: 205-218, 2002.

In addition, some unpublished data are presented.

Abbreviations

AIF	Apoptosis-inducing factor
AP-1	Activating protein-1
AR	Androgen receptor
ArKO	Aromatase knockout
Apaf-1	Apoptotic protease-activating factor-1
ASA	Acetyl salicylic acid
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAD	Caspase-activated DNase
cAMP	Cyclic adenosine monophosphate
CREB	cAMP-response element-binding protein
CREM	cAMP-responsive element modulator
CTL	Cytotoxic T lymphocytes
cyt c	Cytochrome c
DD	Death domain
DED	Death effector domain
Dig-dd-UTP	Digoxigenin-dideoxy-UTP
DISC	Death-inducing signaling complex
DHT	Dihydrotestosterone
DR	Death receptor
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
ERαKO	Estrogen receptor α knockout
ERβKO	Estrogen receptor β knockout
ERαβKO	Estrogen receptor α and β knockout
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain protein
FasL	Fas ligand
FLIP	FLICE inhibitory protein
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin-releasing hormone
hCG	Human chorionic gonadotropin
HSP	Heat shock protein
IAP	Inhibitor of apoptosis protein
IGFBP	Insulin-like growth factor binding protein
IκB	Inhibitor of NF-κB
IKK	IκB kinase
IL	Interleukin
ISEL	*In situ* end labeling
JNK	c-Jun N-terminal kinase
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
NAC	N-acetyl-L-cysteine
NF-κB	Nuclear factor-κB
NK	Natural killer
PBS	Phosphate buffered saline
PLAD	Pre-ligand-binding assembly domain
PMSF	Phenyl methyl sulfonyl fluoride
PT	Mitochondrial membrane permeability transition
p38	p38 kinase
RIP	Receptor interacting protein
ROS	Reactive oxygen species
SCF	Stem cell factor
SMAC	Second mitochondrial activator of caspases
SS	Sulfasalazine
TNFα	Tumor necrosis factor α
TNFR	Tumor necrosis factor α receptor
TRADD	TNFR1-associated death domain protein
TRAF	TNF-receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TNF-related apoptosis-inducing ligand receptor

Abstract

Spermatogenesis is a complex process of male germ cell proliferation and maturation from diploid spermatogonia to haploid spermatozoa that can fertilize the female germ cell and transfer genetic information to the offspring. During spermatogenesis, programmed cell death, i.e. apoptosis, plays an important role in limiting the germ cell population and eliminating germ cells that are defective or that carry DNA mutations. Dysregulation of this physiological germ cell apoptosis can cause male infertility. Inappropriate germ cell apoptosis may also result from external disturbances such as alterations in hormonal support, or exposure to toxic chemicals or radiation. In this respect, exposure to environmental toxicants and hormone-like compounds has been suggested to cause declining sperm counts and male fertility problems. Moreover, survival of cancer patients treated with radiation and chemotherapeutic drugs has increased but the treatments may cause germ cell loss and infertility. Thus, there is a growing need to understand the mechanisms of germ cell death and to find ways to prevent its inappropriate occurrence. Present knowledge of male germ cell apoptosis is based largely on studies conducted in experimental animals, which, in view of potential species specificity of cellular responses to death-inducing stimuli, are not appropriate models for humans.

The present series of studies aimed at characterizing the regulation of the initiating events in human male germ cell apoptosis, using culture of human seminiferous tubules as a model of the physiological stress situation. In this model, exposure of the seminiferous tubules to serum-free culture conditions induced massive germ cell apoptosis within a few hours. The studies specifically addressed *i*) the role of the testicular steroid hormones 17β-estradiol and dihydrotestosterone (DHT) in the regulation of male germ cell death, *ii*) the involvement of the signaling pathways initiated by the cellular death-inducing receptors Fas and the tumor necrosis factor α receptor 1 (TNFR1) in human male germ cell apoptosis, and *iii*) the possibility of preventing stress-induced apoptotic death of male germ cells by pharmacological modulation of the apoptotic pathways characterized in the present studies. The experiments revealed that 17β-estradiol is a survival factor for male germ cells, being an even more potent inhibitor of germ cell death than the androgen DHT. Regulation of germ cell apoptosis was found to involve signaling pathways triggered by the ligands of Fas and TNFR1, i.e. Fas ligand (FasL) and tumor necrosis factor α (TNFα), respectively. While the Fas system appeared to mediate germ cell death, the TNFα-induced signaling was associated with down-regulation of the Fas system and inhibition of germ cell apoptosis. The transcription factor nuclear factor κB (NF-κB), which is often considered to be a mediator of TNFα-induced survival signals, appeared not to mediate the anti-apoptotic effect of TNFα, but rather to be involved in testicular pro-apoptotic pathways that function in parallel with or downstream of that triggered by Fas. Finally, germ cell apoptosis could be prevented by pharmacological modulation of the pathways described in the present studies. Many of the effective compounds are commonly used anti-inflammatory drugs.

In conclusion, the present studies revealed that *i*) physiological estrogens can be considered to be survival factors of male germ cells, which should be taken into account when evaluating the effects on male fertility of compounds able to modulate hormonal signaling, *ii*) the cytokines FasL and TNFα regulate male germ cell death, most likely through activation of their receptors Fas and TNFR1, respectively; FasL mediates germ cell apoptosis and TNFα decreases the level of FasL and inhibits apoptosis, and *iii*) *in vitro*-induced male germ cell apoptosis can be prevented by anti-inflammatory drugs, which raises the possibility of pharmacological suppression of germ cell apoptosis during cancer therapies and other transient stress situations involving excessive germ cell death.

Introduction

Spermatogenesis is a complex process of male germ cell proliferation and maturation from spermatogonia to spermatozoa. During this process, the number of germ cells has to match the nursing capacity of the somatic Sertoli cells, which provide the structural and functional support for germ cell development. In this regard, apoptotic cell death plays an important role in limiting the testicular germ cell population during male development. In the adult testis, also, physiological apoptosis occurs at various phases of spermatogenesis. Dysregulation of germ cell apoptosis, in turn, may cause male infertility. Testicular stress caused by external disturbances, such as alterations in hormonal support or exposure to toxic chemicals or radiation, can cause increased apoptosis leading to pathological germ cell loss. Indeed, exposure to environmental toxicants or chemicals able to modulate hormonal signaling has been suggested to be one reason for declining sperm counts observed in young men. Furthermore, treatment of cancer with radiation or with chemotherapeutic drugs induces germ cell death and may lead to infertility. Accordingly, there is a growing need to study the mechanisms of apoptosis in the testis and to find ways to promote male germ cell survival.

The sensitivity of the seminiferous epithelium to external disturbances such as exposure to radiation, to certain chemotherapeutic compounds, or to *in vitro* culture conditions varies between species. Therefore, results obtained with animal models cannot always be extrapolated to the process of human germ cell death. In the present study, culture of human seminiferous tubules was used as a model for the study of germ cell apoptosis. The culture of seminiferous tubules models the situation in which human testicular homeostasis is threatened and demonstrates how different types of cells in the seminiferous epithelium may act during stress. The present study aimed at characterizing the effects of two testicular steroid hormones, 17β-estradiol and dihydrotestosterone (DHT), on male germ cell death, and the roles of two important induction pathways of apoptosis, the Fas ligand (FasL)- and TNFα-induced pathways, in germ cell apoptosis. Moreover, the present study attempted to find out whether pharmacological modulation of these apoptotic pathways could be used to prevent excessive male germ cell apoptosis.

Review of the Literature

Spermatogenesis

Spermatogenesis is a complex process of male germ cell proliferation and maturation from diploid spermatogonia through meiosis to mature haploid spermatozoa (1). It takes place in the seminiferous tubules of the testis, which consist of (*i*) the seminiferous epithelium, composed of germ cells and supportive somatic Sertoli cells, (*ii*) the basement membrane, and (*iii*) the surrounding peritubular myoid cells (Figure 1A). The interstitial tissue between the seminiferous tubules contains androgen-producing Leydig cells and interstitial macrophages.

Development of the testis

Spermatogonia arise from the primordial germ cells, which migrate into the genital ridge during fetal life (2-4). Under the influence of the Y-chromosome-bearing stromal cells of the developing gonad, they differentiate into gonocytes, the male germ cell precursors, and undergo mitotic arrest (4). After birth, they are reactivated and differentiate into spermatogonia (3,5). In the human testis, the transformation of gonocytes into spermatogonia occurs during the first six months of postnatal life, simultaneously with a transient increase in the serum concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (6). Small numbers of spermatogonia may occasionally differentiate into meiotic primary spermatocytes in the immature human testis, but the vast majority of the germ cells do not undergo meiosis until several years later at puberty (6,7).

Adult spermatogenesis

At puberty, remarkable anatomical, cytological, and functional changes occur in the testis. Sertoli cells cease mitotic divisions, Leydig cells differentiate and produce testosterone in response to LH, and germ cells proliferate intensively and initiate meiosis (7,8). With the initiation of spermatogenesis, groups of germ cells enter the spermatogenic process at regular intervals (5,9). Therefore, germ cells differing in the degree of maturation are not randomly distributed in the seminiferous epithelium, but are arranged in defined associations called stages of the seminiferous epithelial cycle (10). The cycle of the seminiferous epithelium is the time interval between the appearance of the same stage at a certain point of the tubule. The number of stages is constant for a given species. In the human, 6 stages have been defined (Figure 1B) (10). In most species, a particular stage occupies a relatively long segment of a seminiferous tubule, resulting in the appearance of only one stage in a cross-section of a tubule (11). In the human testis, in contrast, the stages are spirally oriented, leading to the typical finding of several irregular cell associations in a given cross-section (12). The duration of the maturation of a spermatogonium to spermatozoa is also species-specific being approximately 70 days in the human testis (12).

Spermatogenesis involves (*i*) spermatogonial proliferation, (*ii*) meiosis, and (*iii*) spermiogenesis (1). Spermatogonia proliferate by mitotic divisions. The germline stem cells known as A_{single} (A_s) spermatogonia, which constitute a minority of the basal germ cells in

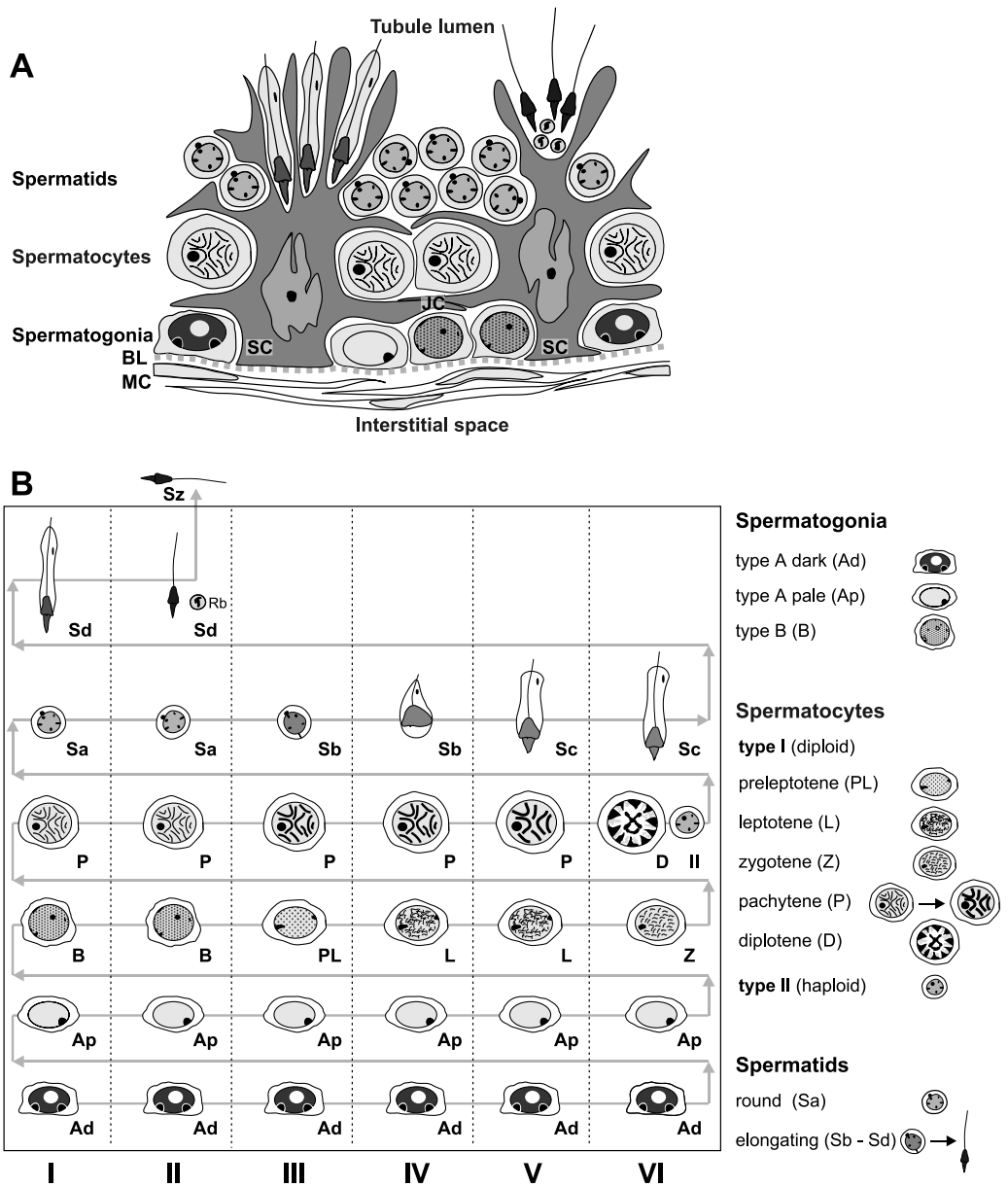

Fig. 1. Human seminiferous epithelium. **A.** Schematic illustration of the structure of human seminiferous epithelium. Germ cells at different phases of differentiation (light gray) are in close contact with the supportive somatic Sertoli cells (SC; dark gray), which form junctional complexes (JC) between each other to produce two compartments of the seminiferous epithelium. The basal compartment below JC contains spermatogonia and early spermatocytes and the adluminal compartment above JC contains later spermatocytes and spermatids. The junctional complexes between Sertoli cells form a barrier that prevents penetration of blood-derived substances into the adluminal compartment where meiosis takes place. The cells of the seminiferous epithelium are separated from the interstitial tissue by the basal lamina (BL) and peritubular myoid cells (MC).

contact with the basement membrane, are single cells that can divide into two new stem cells or into germ cells destinated to differentiate (5,13). From then on, the germ cells consist of interconnected cells of increasing size, because the dividing cells remain connected by cytoplasmic bridges. As germ cell maturation proceeds, these cell syncytia leave the basement membrane and move in a highly ordered manner toward the lumen of the seminiferous tubule. In the human testis, two types of spermatogonia, A spermatogonia and B spermatogonia, are present (12,14,15). Type A_{dark} spermatogonia are thought to be reserve stem cells, with a low division rate (12,14). Type A_{pale} spermatogonia renew more frequently and differentiate into type B spermatogonia, which enter the last mitosis of spermatogenesis and give rise to preleptotene primary spermatocytes (12). This is followed by the long prophase of the first meiotic division, during which homologous chromosomes pair and the primary spermatocytes increase in size and demonstrate distinct nuclear morphology as they pass through the steps of leptotene, zygotene, pachytene, and diplotene (10,12). The first meiotic division is completed after a rapid metaphase, anaphase, and telophase, giving rise to secondary spermatocytes, which undergo the short second meiotic division to form haploid spermatids (12). Finally, during spermiogenesis, a series of transformations of haploid spermatids ultimately leads to the formation of spermatozoa (1,12).

The production of a normal number of spermatozoa depends on the highly specific regulation of gene expression in the germ cells, the paracrine and hormonal control of germ cell proliferation, differentiation, and survival, and the structural and functional support of the germ cells provided by the Sertoli cells (1,9,16-18). Importantly, it is becoming increasingly clear that the specialized functions required for proper proliferation and differentiation of the spermatogonial stem cells are mainly provided by the neighboring differentiated Sertoli cells. The Sertoli cells, possibly together with the adjacent basement membrane, create a particular microenvironment, termed 'niche', which controls the renewal and differentiation of the stem cells (13). Moreover, the cells of the seminiferous epithelium form Sertoli-Sertoli cell and Sertoli-germ cell junctions that mediate adhesive contacts and transmit signals between contiguous cells and that are known to contribute in a crucial way to germ cell maturation (19-22). In the basal region of the seminiferous epithelium neighboring Sertoli cells form specialized junctional complexes, which are composed of *i*) tight junctions, *ii*) unique actin-related dynamic junctions termed ectoplasmic specializations, and *iii*) adhesive intermediate filament-based desmosome-like junctions. These junctional complexes divide the seminiferous epithelium into two distinct microenvironments, a basal compartment that contains spermatogonia and preleptotene

B. Cycle of the human seminiferous epithelium. Germ cells at different phases of differentiation are arranged in six defined cell associations, i.e. stages, of the cycle of the seminiferous epithelium (roman numerals). In humans, the stages are oriented spirally, leading to the occurrence of irregular areas from several different stages in a cross section of the seminiferous tubule. The direction of germ cell maturation from spermatogonia to spermatozoa is indicated by arrows. The excess cytoplasm which is partitioned off from the spermatid in its final phase of maturation is called the residual body (Rb). Thereafter, the germ cell is released from the seminiferous epithelium as a spermatozoon (Sz). The illustrations are based on those of Y. Clermont (10).

spermatocytes and an adluminal compartment that contains later spermatocytes and spermatids. They form a barrier which prevents penetration of many hazardous, blood-derived substances into the adluminal compartment where meiosis takes place. Desmosome-like junctions are also present between the Sertoli cells and the round spermatids. As the spermatids mature, the desmosome-like junctions are replaced by ectoplasmic specializations, which persist until spermiation, when the final junctions between Sertoli cells and germ cells called tubulobulbar complexes form. The turnover of the ectoplasmic specializations enables spermatocytes to move from the basal to the adluminal compartment and the release of sperm. Finally, gap junctions, i.e. intercellular membrane channels that allow cells to communicate directly with one another, are present between the Sertoli cells, between the Sertoli cells and the spermatogonia/ spermatocytes, between the cells of the peritubular layer, and between the Leydig cells. Gap junctions provide a means for the efficient transport of nutrients to cells located far from the blood supply and a conduit for the sharing of small metabolites and second messengers (22-25). Thus, the fine architecture of the seminiferous epithelium and the interactions between different types of seminiferous epithelial cells are crucial for spermatogenesis.

Apoptotic cell death in the testis

Apoptosis

Apoptosis, also known as programmed cell death, is a form of cell death in which the cells activate an intracellular death program and kill themselves in a controlled way, i.e. commit suicide (26). During development, structures that are no longer needed are removed by apoptosis. Throughout life, apoptosis eliminates cells that are useless or potentially dangerous to the host such as aged, infected, injured, or mutated cells, or cells that are produced in excessive amounts, such as germ cells in the testis. During apoptosis, the cells shrink and exhibit several typical features, including cell membrane disruption, cytoskeletal rearrangement, nuclear condensation, and internucleosomal DNA fragmentation (27). The degradation of DNA into fragments approximately 185 bp and its multiples in size is one of the best characterized biochemical features of apoptotic cell death and is used as the basis for the commonly used labeling techniques for detecting apoptotic cells (28). Apoptotic cells are usually rapidly taken up and degraded by neighboring cells before their intracellular contents leak into the extracellular space. In contrast, acute accidental injury may lead to an uncontrolled form of cell death, called necrosis, which is characterized by swelling and bursting of the dying cells, with an accompanying inflammatory response (26).

Caspase activation

Most of the morphological changes in apoptotic cells are caused by specific proteases, caspases, that share the ability to cleave their substrates on the carboxyl side of aspartate residues (27,29). Today, 14 mammalian caspases have been identified of which caspases-2, -3, -6, -7, -8, -9, and -10 have been implicated in apoptosis. The caspases are synthesized as enzymatically inactive zymogens, which in most cases are cleaved proteolytically to produce the active enzyme (30). Caspase activation may result from various intra- and extracellular death-inducing signals and, depending on the cell

type, is mediated via mitochondria-independent or -dependent pathways (Figure 2) (27,31).

In the death receptor pathway, cell-surface death receptors, such as Fas or tumor necrosis factor-α receptor 1 (TNFR1), are activated by ligand binding and recruit several cytoplasmic adapter proteins through homotypic interactions between special interaction domains (27). The resultant membrane-bound signaling complex then recruits several molecules of procaspase-8, which is considered to be the key initiator caspase in the death-receptor pathway (32). Under these conditions, the procaspase-8 molecules are believed to cleave and activate each other (30,32). From this point, the apoptotic signal is mediated via a mitochondria-independent or dependent pathway, depending on the cell type (27,32). In type I cells (e.g. various lymphoid cells), active caspase-8 cleaves large amounts of downstream effector caspases, such as caspase-3, -6, and –7, resulting in effective commitment to apoptosis independently of mitochondrial events. In type II cells (numerous other cells), only small amounts of active caspase-8 are formed and the signal is amplified through activation of the mitochondrial pathway.

The mitochondrial pathway of caspase activation involves mitochondrial events such as membrane permeability transition (PT), with resultant release of mitochondrial proteins such as cytochrome *c* into the cytoplasm (27,33). When released into the cytoplasm, cytochrome *c* binds to Apaf-1 (apoptotic protease-activating factor-1), resulting in the assembly of a high molecular-mass complex called the apoptosome, which contains cytochrome c, Apaf-1, and procaspase-9 (27,34). The interaction between Apaf-1 and procaspase-9 leads to the formation of an active caspase-9 which, in turn, proteolytically activates caspase-3. In the death receptor-initiated pathway of the type II cells, the release of cytochrome *c* from the mitochondria results from caspase-8-mediated cleavage of a cytoplasmic Bcl-2 family member Bid. Bid can also be activated proteolytically by the cytotoxic lymphocyte protease granzyme B and by certain lysosomal cathepsins (27). Importantly, various stimuli can induce PT and thus the release of cytochrome *c* independently of any caspase-8 activation (33).

In both the mitochondria-independent and -dependent pathways, the proteolytic activity of the effector caspases eventually results in the destruction of vital proteins and the death of the cell. In most cases, caspase-mediated cleavage causes inactivation of the substrate proteins, such as polyADP-ribose polymerase, or destruction of macromolecular structures such as the lamin network (30). In addition, caspases activate other proteins that are needed for the achievement of apoptosis by cleavage of regulatory domains. One of these proteins is caspase-activated DNase (CAD), which is responsible for apoptotic DNA fragmentation (28).

Intracellular regulators of apoptosis

Apoptotic pathways are regulated by numerous intracellular factors. The Bcl-2 family of proteins is a major class of intracellular apoptosis regulators (27,35). The Bcl-2 family can be divided into anti-apoptotic members, such as Bcl-2, Bcl-x_L, and Bcl-w, and pro-apoptotic members, such as Bax, Bak, Bid, and Bad. However, recent evidence indicates that caspase-mediated cleavage of the anti-apoptotic Bcl-2 family proteins may convert them into pro-apoptotic mediators (36). It is

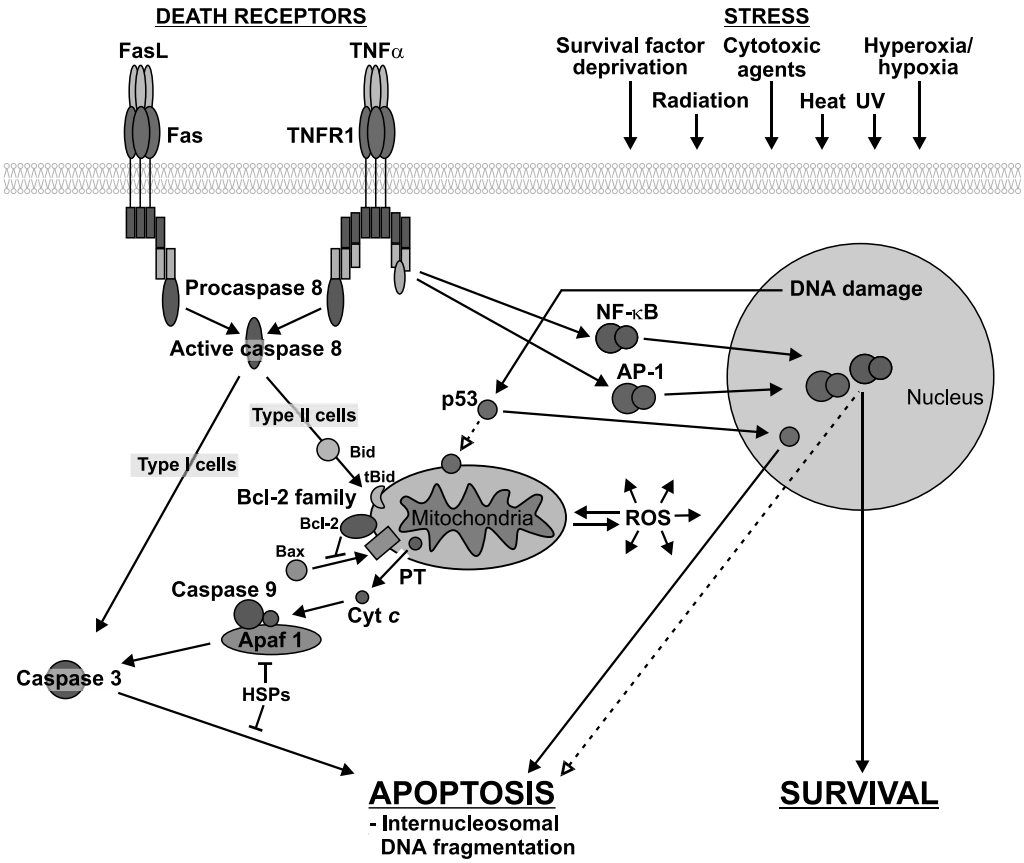

Fig. 2. Multiple pathways of apoptosis. Apoptotic pathways can be initiated by the binding of death-inducing cytokines, such as the Fas ligand (FasL) or the tumor necrosis factor α (TNFα), to their cell membrane receptors, by various other extracellular stress stimuli, or by intracellular stimuli such as DNA damage. Activation of the death receptors is followed by recruitment of several adapter proteins and consequent activation of procaspase-8. A number of intracellular factors, such as transcription factors nuclear factor-κB (NF-κB) and activating protein-1 (AP-1), may be simultaneously induced in response to death-inducing stimuli to regulate the apoptotic pathways. In the mitochondria-independent pathway, activation of caspase-8 leads to direct activation of effector caspases such as caspase-3. In the mitochondria-dependent pathway, caspase-8 cleaves the pro-apoptotic Bcl-2 family member Bid, yielding a fragment (tBid) that translocates into mitochondria, where it takes part in mitochondrial events such as membrane permeability transition (PT) and release of cytochrome *c* (cyt *c*) into the cytoplasm. Various caspase-independent extracellular and intracellular stimuli can also induce these mitochondrial events. Pro-apoptotic Bcl-2 family members, such as Bax, facilitate the release of cyt *c*, whereas anti-apoptotic Bcl-2-like proteins function to prevent their action. The tumor suppressor protein p53 mediates apoptosis by inducing transcription of genes encoding pro-apoptotic proteins, such as Bax and Fas, and possibly also by transcriptionally independent activities, such as relocalization of death receptors from Golgi to the cell surface and direct signaling at the mitochondria. Reactive oxygen species (ROS) formed in the mitochondria during apoptosis can regulate the apoptotic cascade at various levels. Once in the cytoplasm, cyt *c* promotes assembly of procaspase-9 and Apaf-1 into a macromolecular complex called the apoptosome. The anti-apoptotic heat shock proteins (HSPs) may prevent the formation of the apoptosome. Assembly of the apoptosome results in the formation of the active caspase-9 which, in turn, proteolytically activates caspase-3. Thus, the mitochondria-independent and –dependent apoptotic pathways converge at the level

generally believed that the ratio of pro-apoptotic to anti-apoptotic Bcl-2 family members is critical in determining whether the cell will undergo apoptosis. A major function of the Bcl-2 family members appears to be the regulation of mitochondrial events, such as release of pro-apoptotic factors (27,33,35).

Various transcription factors appear to regulate cell death. The tumor suppressor p53 regulates both cell proliferation and apoptosis (37). In response to various stress signals, it can promote apoptosis by enhancing transcription of the genes involved in apoptotic pathways (38,39). However, p53 may also contribute to the regulation of apoptosis by transcriptionally independent activities, such as relocalization of death receptors from Golgi to the cell surface and direct signaling at the mitochondria (38,39). Two other transcription factors, nuclear factor-κB (NF-κB) and activating protein-1 (AP-1), are also known to play roles in both cell proliferation and apoptosis (40-43). Both may be activated in response to various stimuli, such as TNFR1 activation (44,45), and both appear to either promote or inhibit apoptosis, depending on the cell type and the experimental model used (40-42).

In addition, several other intracellular molecules have been shown to regulate apoptosis. Heat shock proteins (HSPs), which accumulate in cells in response to stressful stimuli, can regulate apoptosis by acting as molecular chaperones that influence the assembly of protein complexes, such as the apoptosome, or by protein-protein interactions that are not related to their chaperone function (46,47). In general, HSP27, HSP70, and predominantly also HSP90 are anti-apoptotic, while HSP60 and HSP10 are pro-apoptotic. Furthermore, enzymatic hydrolysis of cell membrane lipids produces bioactive molecules such as the sphingolipids ceramide and sphingosine-1-phosphate (S1P) (48). Several stress agents cause intracellular accumulation of ceramide, which is usually found to be associated with the induction of apoptosis (48,49). S1P has been suggested as an antiapoptotic factor and the balance between the cellular concentrations of ceramide and S1P has been suggested to determine whether the cell will undergo apoptosis or survive (50). Finally, numerous additional intracellular molecules may contribute to the regulation of apoptosis. Among these are cellular caspase inhibitors, such as IAPs (inhibitors of apoptosis proteins) and FLIPs (FLICE, i.e. caspase-8, inhibitory proteins), and polypeptides released from the mitochondria, such as AIF (apoptosis-inducing factor), endonuclease G, and SMAC (second mitochondrial activator of caspases, also called Diablo) (27,32).

Caspase-independent apoptosis

Apoptosis can be induced by overexpression of Bax even when the activity of caspases is blocked, suggesting that caspase activity is not essential in all types of apoptosis (51). Moreover, apoptosis-like chromatin condensation has been reported in some cell types dying in the presence of caspase inhibitors (52). As Bax is known to induce PT, the energy depletion and generation of reactive oxygen species that accompany PT have been

of caspase-3. Degradation of various cellular structures by caspase-3, by other downstream effector caspases, and by other enzymes activated by caspase-3 lead to typical features of apoptosis, such as internucleosomal DNA fragmentation.

suggested to explain this caspase-independent cell death (27). Endonuclease G, which is also released from mitochondria, has been proposed to account for the apoptosis-like chromatin condensation in cells dying by caspase-independent apoptosis (27).

Germ cell apoptosis

Physiological germ cell apoptosis

During prepubertal development, a wave of extensive germ cell apoptosis is observed in the rodent testis (53,54). This early germ cell apoptosis, which mainly affects spermatogonia and spermatocytes, appears to be essential for functional spermatogenesis in adulthood. In the adult testis also, normal spermatogenesis is accompanied by spontaneous germ cell degeneration, which appears to be mainly apoptotic and to result in the loss of up to 75% of the potential number of mature spermatozoa (55-58). In the human testis, spontaneous germ cell apoptosis involves all three classes of germ cell, i.e. spermatogonia, spermatocytes, and spermatids (58). However, the exact incidence of adult male germ cell apoptosis is unclear, because only the spermatogonia and round spermatids display the classical morphological and biochemical features of apoptosis (3). Identification apoptotic spermatocytes and elongated spermatids is less clear, because of their unusual morphology and DNA configuration (3).

The physiological significance of the spontaneous germ cell apoptosis that occurs during spermatogenesis is unclear. Since Sertoli cells are terminally differentiated cells with no capacity for renewal, and are able to support only a certain number of germ cells, the number of maturing germ cells must be limited (8). Therefore, germ cell death, at least during development, most likely occurs in order to limit the number of germ cells to match the supportive capacity of the Sertoli cells (3,53). Apoptosis may also serve to eliminate germ cells with altered DNA (53). In meiotic spermatocytes, there appears to be a quality-control system or checkpoint for monitoring chromosome synapsis (59). This control system is thought to recognize unrepaired double-strand DNA breaks in unsynapsed chromosomes during meiotic metaphase and to induce apoptosis of the affected cell. Whether unrepaired DNA breaks are the only inducers of apoptosis of cells containing unsynapsed chromosomes is not known. Surprisingly, the quality control-induced apoptosis of meiotic germ cells appears to be independent of p53, which is a major regulator of apoptosis in response to DNA damage in somatic cells and is strongly expressed in pachytene spermatocytes (60).

Inappropriate germ cell apoptosis

Inappropriate male germ cell apoptosis is associated with pathological conditions such as infertility, cryptorchidism, and testis torsions (53,61-65). Moreover, in rodents, increased germ cell death has been shown to be induced *in vivo* by external disturbances, such as alterations of hormonal support, toxicant exposure, or radiation (66-70). Consistently, massive germ cell apoptosis occurs *in vitro* in human seminiferous tubules cultured under serum-free conditions, the apoptotic cells being mainly pachytene spermatocytes and round spermatids (71). Notably, in cultured rat seminiferous tubules, apoptotic DNA fragmentation is not found until 24 hours of culture (72, our unpublished observations) but in human seminiferous tubules is already

evident after a few hours (71) indicating species specificity in the sensitivity of testicular germ cells to external death-inducing stimuli.

Defective spermatogenesis and male infertility have been observed when expression of apoptosis-related genes is disrupted or inappropriately controlled (17,53,58,73). Thus, high levels of the pro-apoptotic Bcl-2 family members Bax and Bak in relation to the anti-apoptotic Bcl-2 family members are associated with germ cell apoptosis during development and their imbalance results in infertility (53,54,73). Moreover, the anti-apoptotic Bcl-2 family member Bcl-w appears to be an important survival factor for Sertoli cells, spermatogonia, and spermatocytes (74) and its deficiency leads to increased postpubertal Sertoli and germ cell death and consequent disruption of spermatogenesis (75-77). Furthermore, the tumor suppressor p53 is involved in radiation- and cryptorchidism-induced mouse germ cell apoptosis (78-80) and its overexpression in mice results in increased germ cell apoptosis and decreased production of spermatozoa (81). In addition, mice with disruptions of various other genes, including those encoding the transcriptional activator CREM, the testis-specific heat-shock protein HSP70-2, and several other proteins involved in DNA repair and cell-cycle control, exhibit defective spermatogenesis and increased germ cell apoptosis (17,58,82).

Hormonal control of germ cell apoptosis

Gonadotropins and androgens

Gonadotropins and testosterone have been shown to regulate testicular germ cell apoptosis in a stage-specific manner (6,58). Thus, in the immature rat, hypophysectomy or treatment with gonadotropin-releasing hormone (GnRH) antagonist results in increased germ cell apoptosis, which can be inhibited by human chorionic gonadotropin (hCG) or testosterone (70,83). Apoptosis in the immature rat testis can also be induced by immunoneutralization of FSH (84). In the adult rat, gonadotropin withdrawal by GnRH antagonist-treatment or immunoneutralization of FSH appears to result in increased germ cell apoptosis, mainly affecting the pachytene spermatocytes and spermatids (68,84,85). Consistently, in cultured adult rat seminiferous tubules, apoptosis of pachytene spermatocytes and spermatids can be inhibited by FSH (72). Thus, rodent studies support an anti-apoptotic role of gonadotropins in the testis. However, in prepubertal boys with cryptorchid testes, treatment with hCG results in increased spermatogonial apoptosis (61). This germ cell loss is associated with reduced testis volume and lowered sperm counts in adulthood (61).

The importance of testosterone in the regulation of germ cell apoptosis in the adult rat testis has been shown in experiments in which decreased serum and intratesticular concentrations of testosterone were caused by *in vivo* destruction of Leydig cells (86,87). Testosterone withdrawal induces apoptotic cell death in most stages of the cycle and appears to mainly affect spermatocytes and spermatids. These effects can be suppressed by testosterone supplementation (86). Interestingly however, testosterone seems to be pro-apoptotic at one stage of the cycle (86). Consistently with these results of rodent studies, experiments conducted in our laboratory have revealed that testosterone is able to effectively inhibit *in*

vitro-induced apoptosis of human spermatocytes and spermatids and is thus a germ cell survival factor also in the human testis (71).

The mechanisms by which gonadotropins and androgens regulate germ cell apoptosis are unclear. The receptors for LH/hCG are expressed in Leydig cells, which, under the influence of LH, secrete testosterone (88). Furthermore, in most studies the receptors for androgens have been found in the Sertoli, peritubular myoid, and Leydig cells, and those for FSH in the Sertoli cells (88,89). Thus, according to these studies, germ cells seem to be devoid of receptors for gonadotropins and androgens, which suggests that these hormones act by a paracrine mechanism. The antiapoptotic effect of FSH appears to be partially mediated via the stem cell factor (SCF), which is produced by Sertoli cells and interacts with the c-kit receptor on germ cells (90). This mechanism may involve changes in the Bcl-2 family members, since, in cultured rat seminiferous tubules, either FSH or Sertoli cell-derived SCF can up-regulate the anti-apoptotic Bcl-w (54,74). The Bcl-2 family may also contribute to the anti-apoptotic effect of testosterone, as *in vivo*-induced testosterone withdrawal and consequent germ cell apoptosis in the rat is associated with down-regulation of Bcl-w and up-regulation of pro-apoptotic Bax and Bak (74,87). Importantly, testosterone can be metabolized *in vivo* to either estrogens or dihydrotestosterone (DHT), both of which may, at least to some extent, mediate the pro-survival effects of testosterone.

Estrogens

In addition to the established role of gonadotropins and androgens in spermatogenesis and testicular apoptosis, estrogens are now recognized as potential regulators of male reproduction and germ cell death (91,92). Estrogens are formed from testosterone by the enzyme P450 aromatase, which is present in the Sertoli cells of the immature testis and the Leydig cells of the adult testis (92). In several species, P450 aromatase is also expressed in the germ cells (92-95). Estrogens can cause alterations in the circulating concentrations of gonadotropins and testosterone (92) and can thus affect germ cell apoptosis indirectly. In addition to their systemic effects, estrogens have specific direct effects in the male reproductive tract. At least some of these appear to be mediated by local estrogen receptors (ERs), which exist in at least two subtypes, ERα and ERβ. Testicular expression of the ERs has been shown in numerous recent reports, but the results regarding their cellular localization are controversial (92). Moreover, the expression patterns of the ERs seem to be species specific (92). In most studies, ERs have been found in Sertoli and Leydig cells and in germ cells from pachytene spermatocytes to round spermatids. The subtype of ER in the seminiferous epithelium has most often been found to be ERβ. In addition to the conventional ERs, recent data suggest the presence of a functional membrane-associated ER on human spermatozoa, which, when activated by 17β-estradiol, appears to induce a nongenomic signaling pathway (96).

Direct evidence for a role of estrogens in male germ cell survival has been obtained from studies on mice deficient in functional ERα (ERα knockout, ERαKO) (97,98), aromatase (ArKO) (99), ERβ (ERβKO) (100), or both ERs (ERαβKO) (101). The ERαKO males are infertile because of impaired reabsorption of fluid in the efferent ductules and resultant pressure-induced

atrophy of the seminiferous epithelium (97,98). Interestingly, the ArKO mice develop progressive disruptions of spermatogenesis and infertility, but they have no abnormal fluid reabsorption in the efferent ductules(99). Instead, the defective spermatogenesis in the ArKO mice appears to be caused by a direct effect of estrogen withdrawal on the seminiferous epithelium and to involve increased germ cell apoptosis (99). Somewhat surprisingly, the ERβKO mice are fertile and have no apparent disruption of spermatogenesis (100). Moreover, the ERαβKO appear to have a similar phenotype to ERαKO mice (101) suggesting that ERβ does not play a major role in the seminiferous epithelium.

In man, the importance of estrogens for normal human spermatogenesis is suggested by case reports of two men, one with a homozygous inactivating mutation in the ERα gene (102) and the other with the P-450 aromatase gene (103). The patient with the mutation in the ERα gene had normal male genitals and sperm density, but sperm viability was severely decreased. The mutation in the aromatase gene resulted in infertility, with a decreased sperm count and 100% immotile spermatozoa. However, the exact roles of estrogens in human spermatogenesis have remained unknown.

Apoptosis control by the death receptors Fas and TNFR1

Death receptors and ligands

Death receptors are cellular receptors which, after binding of specific death ligands, can activate caspases within seconds and cause apoptotic death of the cell within hours. Death receptors belong to the large TNFR superfamily (104,105). Members of this protein family are characterized by similar cysteine-rich extracellular domains. A subset of these proteins, namely Fas (CD95/Apo-1), TNFR1 (p55/CD120a), death receptor 3 (DR3; TRAMP/Apo3/WSL-1/LARD), TRAIL-R1 (DR4), TRAIL-R2 (DR5/Killer/TRICK2), and DR6, contains an intracellular "death domain" (DD), and is therefore called the death receptor subfamily (44,105). DD is an 80-amino-acid-long region that is essential for transduction of the apoptotic signal (32). The ligands that bind to the death receptors are structurally related proteins that belong to the TNF superfamily (44,105). These include FasL (CD95L), TNFα, lymphotoxin α, TWEAK (Apo3 ligand), and TRAIL (Apo2 ligand). The best-characterized death receptors/ligands are Fas/FasL and TNFR1/TNFα.

Fas is a widely expressed glycosylated type I transmembrane protein with a relative molecular mass of approximately 45 to 52 kDa (106,107). In addition, a soluble form generated by alternative mRNA splicing may regulate Fas-mediated apoptosis (108,109). The natural ligand for Fas is FasL, which is a 40 kDa type II membrane protein (110) that may also be cleaved by a metalloproteinase to produce a soluble ligand (111-113). FasL is expressed in a more restricted way than Fas and is predominantly found in activated lymphocytes and Natural Killer cells (114). The testis is a major nonlymphoid site of FasL expression (110,115).

TNFα has two receptors, TNFR1 (p55) and TNFR2 (p75), of which only TNFR1 contains the cytoplasmic DD and belongs to the family of death receptors. TNFR1 is a transmembrane receptor protein with a predicted molecular

mass of approximately 45 kDa and, after glycosylations, of 55 to 60 kDa (116). It is widely expressed by somatic cells and can be cleaved by matrix metalloproteinases to produce a soluble receptor. TNFα, in turn, is a potent cytokine that is produced by many cell types in response to inflammation, infection, or injury (117). The TNFα protein is formed as a 26 kDa membrane-bound precursor, which is cleaved by a metalloproteinase to generate the secreted 17 kDa mature cytokine (118,119).

Mechanisms for the regulation of apoptosis by Fas and TNFR1

In death receptor-mediated apoptosis, aggregation of the cellular death receptors with their natural ligands or agonistic antibodies activates the apoptotic death machinery in the receptor-bearing cells (44,114,120). Each ligand can bind three receptors, and trimerization is most likely needed for transduction of the apoptotic signal. Recently, another model has been suggested, in which extracellular pre-ligand-binding assembly domains (PLADs) aggregate the receptors before ligand binding, and premature signaling is prevented by intracellular receptor-associated apoptosis blockers (121,122). The intracellular DDs of the aggregated receptors bind to specific signaling molecules, which either link the receptors to the apoptotic caspase pathway or, especially in the case of TNFR1 activation, may mediate functions that are distinct from or even counteract apoptosis (Figure 3).

Fas activation (Figure 3) leads to association of a complex of proteins, the death-inducing signaling complex (DISC), with the activated receptor (32,123). DISC involves FADD (MORT1), which binds via its own DD to the DD of Fas, and procaspase-8 (FLICE/MACH), which is recruited by FADD via interaction of death effector domains (DEDs) that are present in both FADD and procaspase-8. Various other proteins have also been said to bind to the activated Fas and to DISC, but their role in the regulation of Fas-mediated apoptosis remains to be defined (32,124). Procaspase-8 is activated proteolytically and released from the DISC into the cytoplasm. As described above in the paragraph “Apoptosis”, activation of caspase-8 may, depending on the cell type (type I or II), lead either to direct activation of the downstream effector caspases or, more often, to amplification of the signal by activation of the mitochondrial pathway (27,31,32). Under some circumstances, activation of a phosphorylation-based signaling pathway involving a c-Jun amino-terminal kinase (JNK) subgroup of a family of mitogen-activated protein kinases (MAPKs) may contribute to Fas-mediated apoptosis (125,126). Sustained activation of JNK by Fas or by other stimuli appears to be associated with apoptosis, whereas transient JNK activation is usually associated with the induction of survival pathways (125,126). Thus, activation of JNK by Fas may not only favor apoptosis, but may also cause cellular events involved in adaptation to stress (127). In some types of cell, Fas can also induce an extracellular signal-regulated kinase (ERK) subgroup of MAPKs that mediate cell survival and protect from Fas-induced apoptosis (128,129).

TNFR1 activation can also result in activation of the caspase cascade leading to apoptosis (Figure 3). However, TNFR1 also mediates activation of transcription factors such as NF-κB and AP-1, which can induce genes involved in the suppression of apoptosis (Figure 3) (44,45). Binding of TNFα to TNFR1 results in

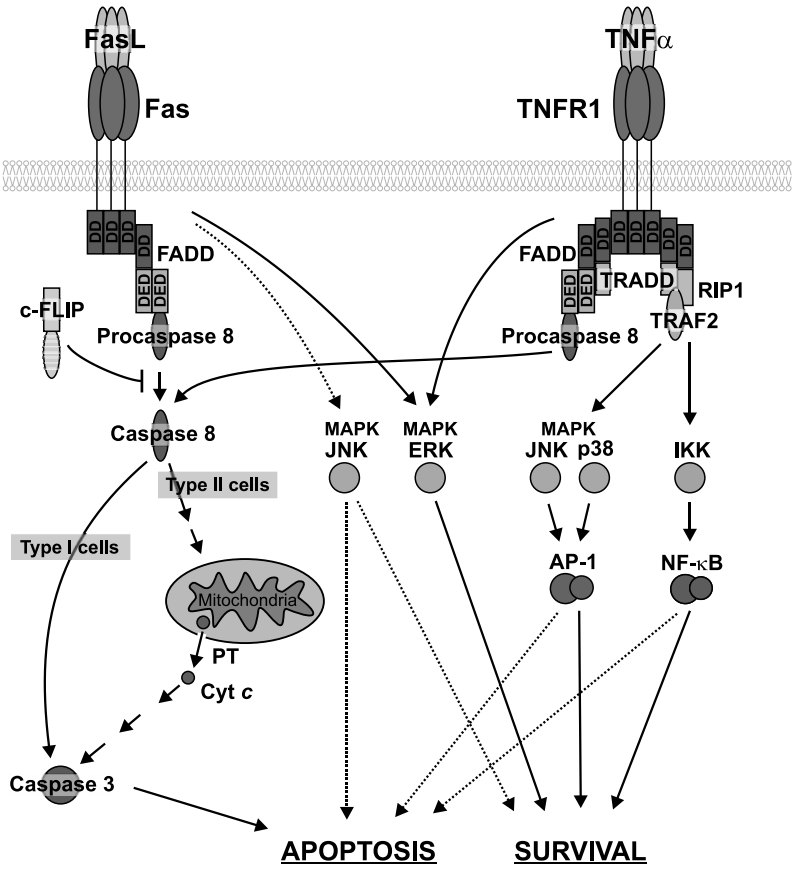

Fig. 3. Pro- and anti-apoptotic signaling pathways induced by Fas and TNFR1. FasL or TNFα binding to the death receptors Fas or TNFR1, respectively, results in receptor aggregation and recruitment of adapter proteins. Both receptors transduce apoptotic signals through the recruitment of FADD and subsequent recruitment and activation of procaspase-8. This is followed by direct activation of caspase-3 (type I cells) or amplification of the signal in the mitochondria (type II cells). Caspase-8 activation can be blocked by recruitment of the caspase-8-like inhibitory protein c-FLIP. In some cell types, the JNK subgroup of MAPKs may mediate the death signal from Fas, but the mechanism of Fas-induced JNK activation is not clear. TNFR1 recruits TRADD, which binds the apoptosis-mediating FADD. TRADD also binds RIP1 and TRAF2, which mediate activation of the transcription factors AP-1 and NF-κB through activation of the protein kinases JNK, p38, and IKK. AP-1 and NF-κB are usually involved in cell survival, but, depending on the cell type, may also mediate apoptosis. In some cell lines, both Fas and TNFR1 may activate MAPK/ERK, which protect the cell from death receptor-induced apoptosis.
Abbreviations: TNFR1, tumor necrosis factor α receptor 1; FasL, Fas ligand; TNFα, tumor necrosis factor α; DD, death domain; DED, death effector domain; FADD, Fas-associated death domain protein; TRADD, TNFR1-associated death domain protein; JNK, c-Jun N-terminal kinases; MAPK, mitogen mitogen-activated protein kinases; RIP1, receptor-interacting protein 1; TRAF2, TNF-receptor-associated factor 2; AP-1, activating protein-1; NF-kB, nuclear factor-κB; p38, p38 kinase; IKK, IκB kinase; ERK, extracellular signal-regulated kinase; PT, permeability transition; cyt *c*, cytochrome *c*.

receptor trimerization and recruitment of a DD-containing protein TRADD (TNFR1-associated death domain protein). TRADD binds FADD via the interaction of the DDs, which leads to activation of the procaspase-8 as in Fas-mediated signaling. However, TRADD also binds at least two additional mediators, RIP1 (receptor-interacting protein 1) and TRAF2 (TNF-receptor-associated factor 2), which stimulate pathways leading to activation of NF-κB (via IκB kinase) and AP-1 (via JNK or p38 kinase subgroups of MAPKs). In addition, TNFR1 may induce activation of MAPK/ERK that protect from apoptosis (129). Fas does not bind with high affinity to effector molecules such as TRADD, RIP1, or TRAF2, and is therefore a poor activator of NF-κB and AP-1 and more effective than TNFR1 for the induction of apoptosis (45,124).

Physiological roles of the FasL- and TNFα- induced signaling

FasL-induced signaling is suggested to play a major role in several types of physiological apoptosis (44,114,123,124,130). First, Fas initiates activation-induced death of lymphocytes, which is essential for down-regulation of the immune reaction and protection against autoimmunity. To downregulate the immune reaction, activated T lymphocytes, which express both Fas and FasL, either undergo Fas-mediated suicide, or kill each other. Loss-of-function mutations in the *Fas* or *FasL* genes result in accumulation of mature T cells in the lymph nodes and spleen of mice and humans. Second, cytotoxic T lymphocytes (CTL) and natural killer (NK) cells kill infected cells via Fas-mediated apoptosis. When activated through T cell-receptor interaction with viral antigens, the cytotoxic cells start to express FasL and kill the antigen-presenting cells that express Fas. Inappropriate FasL-mediated cytotoxic action of the CTL results in tissue destruction such as occurs in hepatitis and graft-vs-host disease. Third, killing of inflammatory cells at immune-privileged sites such as the eye and the testis has been suggested to be mediated by the Fas system. According to this model, stromal cells of the immune-privileged tissues constitutively express FasL, which kills Fas-expressing inflammatory cells. Similarly, tumor cells have been suggested to escape from the immune system by expressing FasL. However, recent studies have shown that FasL is expressed on CTL after tumor recognition and that the CTL are killed not by tumor cells but by themselves and by neighboring CTL (131). The role of FasL as a mediator of the immune-privileged nature of the testis has also been questioned (131).

TNFα-induced signaling is involved not only in apoptosis but also in inflammatory responses and in cell proliferation and differentiation. The primary sources of TNFα are activated macrophages, but its production is also induced in many other cell types in response to various environmental factors (45,132). The major role of TNFα is to provide protection against infections and tumors (117,132). At the level of the individual cell, TNFα may induce death of an infected or transformed cell and, at the level of multicellular organs and the whole organism, it mediates inflammatory responses such as lymphocyte and leukocyte activation and migration, fever, and elevation of acute-phase serum proteins (117,132,133). The broad spectrum of the effects of TNFα is explained by the ability of the TNF receptors to mediate the activation of the transcription factors AP-1 and NF-κB, which, in turn, commonly induce genes

involved in inflammatory responses and cell proliferation (43,45,134). AP-1 and, more commonly, NF-κB can also induce genes encoding proteins that mediate suppression of TNFα-induced apoptosis. Thus, although TNFα induces apoptosis in transformed or infected cells, in normal cells cell death is a rare response to TNFα and usually occurs only when gene expression is inhibited by RNA or protein synthesis blockade (45,135-137).

The Fas system in the testis

The Fas system has been suggested to play a role both in maintaining the immune-privileged nature of the testis and in regulating testicular germ cell apoptosis. FasL has been found in mouse, rat, and human Sertoli cells (87,115,138,139) and is generally assumed to be constitutively expressed by the Sertoli cells. Some reports have shown FasL in germ cells also (87,138). Fas expression, in turn, has been demonstrated in the germ cells of the rat and human testes (87,138,140), and in some reports also in the Sertoli cells (87,138,141).

The idea of FasL as a mediator of testicular immune-privilege is based on the finding that testis grafts from normal mice survive when transplanted under the kidney capsule of allogenic animals, whereas testis grafts from mice deficient in functional FasL are rejected (115). FasL, which was found to be expressed in the Sertoli cells of the normal mice, was suggested to induce apoptosis of Fas-expressing recipient T-cells activated in response to graft antigens. This led to the suggestion that expression of functional FasL by Sertoli cells accounts for the immune-privileged nature of the testis. However, this concept will have to be re-evaluated, because, in more recent studies, FasL expression on other transplants caused inflammation and rapid rejection instead of graft survival (131).

The role of the Fas system in testicular germ cell apoptosis is supported by several findings in rodent models. In the rat testis, the expressions of both Fas and FasL are up-regulated concomitantly with increased germ cell apoptosis after exposure of the animals to Sertoli cell toxicants (140). Furthermore, mouse germ cells *in vitro* are susceptible to anti-Fas antibody-induced death and the survival of cultured rat germ cells is increased when the expression of FasL is blocked by antisense oligonucleotide treatment (140). Radiation exposure, which primarily targets the actively dividing germ cells without causing damage to the Sertoli cells, increases Fas but not FasL in the rat testis (142). In contrast, exposure of rats to Sertoli cell toxicants results in testicular up-regulation of FasL, followed by up-regulation of Fas (142). These results suggest that *i*) the Fas system mediates testicular germ cell apoptosis, *ii*) Fas up-regulation takes place at the initiation of male germ cell death, and *iii*) Sertoli cell injury causes up-regulation of FasL, which eliminates Fas-positive germ cells. However, there have been no studies concerning the function of the Fas system in the human testis.

Testicular production and effects of TNFα

In the testis, TNFα is produced by the germ cells and is held to be one of the testicular paracrine factors that regulate spermatogenesis. In mouse seminiferous tubules, TNFα is mainly produced by the round spermatids (143). In addition, activated interstitial macrophages of the mouse and rat testis have been shown to

secrete TNFα (144,145). TNFR1, in turn, has been found in the Sertoli and Leydig cells of the mouse and of the porcine testis (143,146-148). Several effects of TNFα on these somatic cells have been documented. In the Sertoli cells, TNFα induces IL-6 production and adhesion molecule expression (146,149,150). It has also been suggested to play a role in the local control of spermatogenesis, because in the Sertoli cells it regulates the production of lactate (151,152), transferrin (153), cAMP-response element-binding protein (CREB) (154), and insulin-like growth factor binding protein (IGFBP) (155). In addition, cultured Leydig cells have been shown to respond to TNFα by decreasing the biosynthesis of testosterone (148,156,157).

In cultured mouse Sertoli cells, TNFα regulates the expression and function of the Fas system, suggesting a role for this cytokine in testicular apoptosis (141). The cultured Sertoli cells express low levels of functionally active membrane-bound Fas protein, which are markedly increased by stimulation with TNFα (141). Therefore, inflammatory cytokines have been suggested to create a proapoptotic environment by inducing up-regulation of Fas in Sertoli cells, which leads to Sertoli cell death when contact occurs with FasL-bearing inflammatory cells. On the other hand, TNFα, at concentrations lower than those needed for induction of the membrane-bound Fas, induces a soluble anti-apoptotic form of Fas (141). Therefore, *in vivo* TNFα produced by germ cells may induce the soluble Fas, which is a potential survival factor in the seminiferous tubules. However, although studies on rodent Sertoli and Leydig cells offer valuable information on the possible roles of TNFα in regulating spermatogenesis and testicular cell apoptosis, the effects of this cytokine on maturing germ cells has previously remained unknown. Moreover, no previous reports have considered the effects of TNFα on germ cell apoptosis in the human testis.

Nuclear factor κB (NF-κB)

NF-κB/Rel and IκB proteins

NF-κB is a dimeric DNA sequence-specific transcription factor which is assembled from two of the five known mammalian Rel/NF-κB proteins, i.e. RelA/p65, RelB, c-Rel, p50 and p52 (134). In most cells, the major Rel complex is the p50-RelA heterodimer. In unstimulated cells, NF-κB proteins remain sequestered in the cytoplasm by inhibitory IκB proteins that include p105, IκBγ, p100, IκBα, IκBβ, and IkBε (158,159). These inhibitory proteins have different affinities for individual NF-κB complexes, are regulated differently, and are expressed in a tissue- and cell-specific manner. The proteins p105 and p100 are inactive precursor forms of p50 and p52, respectively, and require post-translational processing to produce the active NF-κB subunits. IκBγ corresponds to the C-terminal domain of p105. IκBα, IκBβ, and IkBε bind to certain NF-κB complexes and prevent nuclear translocation and DNA binding by covering the nuclear localization sequence of NF-κB and by interfering with sequences important for DNA binding. In addition, there exists a nuclear IκB protein, Bcl-3, which can complex with specific NF-κB dimers and activate κB-dependent transcription.

Activation and target genes of NF-κB

NF-κB can be activated by a variety of extracellular stimuli, including various microbial

products, inflammatory cytokines, physical stress, oxidative stress, mitogens, growth factors and hormones, drugs, and hazardous environmental compounds (43). Most signals that lead to activation of NF-κB induce a common pathway involving phosphorylation and proteosome-mediated degradation of IκB (Figure 4) (160). The key step in this pathway is activation of a high-molecular-weight IκB kinase (IKK) complex that contains two related catalytic kinases, IKKα and IKKβ, and a regulatory polypeptide IKKγ. When activated, the IKK complex catalyzes the phosphorylation of two conserved serines in the N-terminal regulatory domain of IκBs. This is followed by ubiquitination of the phospho-IκB, which targets it for proteosomal degradation. The liberated NF-κB then rapidly translocates into the nucleus, where it regulates transcription by binding to 10-base-pair DNA sites, *i.e.* consensus κB sites, in the promoters of the target genes (43).

NF-κB transcription factors bind κB sites as dimers. Because the different NF-κB proteins can form various homodimers or heterodimers and the individual dimers have distinct DNA-binding specificities, the NF-κB transcription factors regulate a variety of genes (43). These include genes encoding a number of cytokines and their modulators, immunoreceptors, proteins involved in antigen presentation, cell adhesion molecules, acute phase proteins, stress response proteins, cell-surface receptors, growth factors, transcription factors, and enzymes. Because NF-κB activity is induced during various stress conditions and many of the genes induced by NF-κB are involved in the regulation of immune, inflammatory, and stress responses, NF-κB is considered to be a central regulator of stress responses. One of the genes induced by NF-κB is that encoding IκBα, the best known IκB protein. Newly synthesized IκBα can enter the nucleus, remove NF-κB from the DNA, and export the complex back into the cytoplasm (Figure 4) (161,162). In this way, NF-κB limits its own activation.

Despite the large number of genes that can be induced by activated NF-κB, the response is usually specific. This can be explained by *i*) selective activation of NF-κB proteins, and *ii*) the requirement of more than one transcription

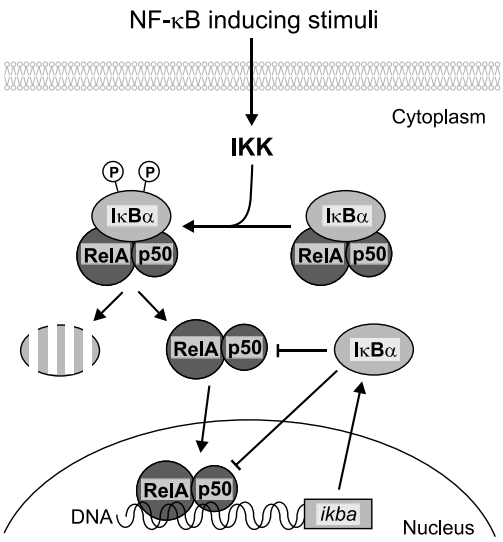

Fig. 4. NF-κB activation. In unstimulated cells, NF-κB (the heterodimer of RelA and p50 in the figure) is retained in the cytoplasm by inhibitory IκB (IκBα in the figure). Various NF-κB-inducing signals cause activation of the IκB kinase complex (IKK). IKK phosphorylates IκB, which leads to its proteosomal degradation. Liberated NF-κB translocates into the nucleus, where it binds to consensus κB sites in the promoters of the target genes and regulates gene expression. One of the target genes of NF-κB is that encoding IκBα. The newly synthesized IκBα may bind NF-κB in the cytoplasm or enter the nucleus, remove NF-κB from the DNA, and export the complex back into the cytoplasm. In this way, NF-κB may limit its own activation.

factor to induce effective transcription of a given gene (43). Thus, the presence of different NF-κB dimers, the synthesis and activation of which may be controlled by distinct pathways, increases the selectivity of the NF-κB-mediated gene transcription. Moreover, NF-κB activity is necessary but may not alone be sufficient for the full transcription of certain genes, and thus other transcription factors activated under specific circumstances may also be required.

Regulation of apoptosis by NF-κB

NF-κB transcription factors may have both anti-apoptotic and pro-apoptotic effects (40). The anti-apoptotic activities of NF-κB have been observed in certain non-testicular cells in response to certain external stimuli, such as TNFα, ionizing radiation, and chemotherapeutic compounds (163-166). The inhibitory effect of NF-κB on TNFα- or chemotherapy-induced apoptosis has also been shown in chemotherapy-resistant tumors (167). On the other hand, there is growing evidence for the apoptosis-promoting functions of NF-κB. In human embryonic kidney cells, serum withdrawal induces NF-κB activation and apoptosis, which can be prevented by overexpression of a dominant negative form of RelA (168). Double-positive ($CD4^+CD8^+$) T cells from mice overexpressing a superinhibitory mutant form of IκBα are resistant to activation-induced cell death (169). Furthermore, NF-κB stimulates the expression of the death-promoting FasL in T cells following T-cell receptor engagement or exposure to DNA-damaging agents, thus suggesting a pro-apoptotic role for NF-κB (170,171). Interestingly, a recent report suggested that during the onset of inflammation, NF-κB activation is associated with the expression of pro-inflammatory and anti-apoptotic genes, whereas during resolution of the inflammation, such activation is associated with the expression of anti-inflammatory genes and the induction of apoptosis (172). Thus, at the onset of carrageenin-induced pleurisy in the rat, *in vivo* inhibition of NF-κB reduced leukocyte expression of the pro-inflammatory cytokines lymphotoxin B and TNFα and of the anti-apoptotic protein Bcl-2. However, during resolution of the inflammation, inhibition of NF-κB resulted in down-regulation of pro-apoptotic Bax and p53 and in concomitant inhibition of leukocyte apoptosis. Finally, recent evidence indicates that NF-κB may have either pro- or anti-apoptotic effects in the same cell type, depending on the death-inducing stimulus (173). Taken together, whether NF-κB promotes or inhibits apoptosis appears to depend on the specific cell type and the type of the inducer. Therefore, to understand the role of NF-κB in different physiological situations, the behavior of this transcription factor in different models of apoptosis needs to be characterized.

NF-κB in the testis

Recent data have suggested a role for NF-κB in regulating rodent spermatogenesis. In the rat testis, the NF-κB complex of RelA and p50 proteins is constitutively expressed in the nuclei of Sertoli cells at all stages of spermatogenesis, but in some stages the expression is higher than in others (174). In addition, nuclear NF-κB is present in a stage-specific manner in pachytene spermatocytes and round spermatids (174). Moreover, in cultured rat Sertoli cells, an increase in nuclear NF-κB DNA binding activity and in κB-dependent transcription can be induced by TNFα (174). As TNFα is known to be secreted by round spermatids (143), a paracrine mechanism has been suggested, in which germ cell-derived TNFα modulates

spermatogenesis by activating Sertoli cell NF-κB (175). In accord, TNFα-induced activation of NF-κB in rat Sertoli cells *in vitro* leads to up-regulation of the cAMP-response element-binding protein (CREB), which is an important regulator of a number of cAMP-induced genes and consequently has been suggested to be a regulator of spermatogenesis (154). However, the physiological role of NF-κB in the testis has still remained unclear.

Aims of the Study

Germ cell apoptosis is essential for normal spermatogenesis and its dysregulation may lead to male infertility. Thus, understanding the causes and mechanisms of germ cell apoptosis is of major importance in preventing male reproductive problems. Prior to the present study, knowledge on the regulation of male germ cell apoptosis was mainly based on investigations conducted in experimental animals. In view of species specificity of cellular responses to death-inducing stimuli, a human model was also required. Such a model, in which germ cell apoptosis is induced in a culture of human seminiferous tubules, was recently created in our study group.

The present series of studies aimed at characterizing the regulation of the initiating events in human male germ cell apoptosis, using the culture of human seminiferous tubules. In particular, elucidation of the following issues were addressed:

1. The effects of the physiological steroid hormones 17β-estradiol and DHT on male germ cell death,

2. The roles of Fas- and TNFR1-mediated signaling and the closely related NF-κB activation pathway in human testicular germ cell apoptosis,

3. The possibility of prevention of stress-induced apoptotic death of male germ cells by pharmacological modulation of the apoptotic pathways characterized in the present studies.

Materials and Methods

Patients

Testis tissue was obtained from 45 men aged 59 - 88 years undergoing orchidectomy as treatment for prostate cancer. They had not received hormonal, chemotherapeutic, or radiotherapeutic treatment for the cancer before the operation. They had no endocrinological disease and none of them had suffered from cryptorchidism. The operations were performed between September 1997 and January 2001 at the Department of Urology, Helsinki University Central Hospital (Helsinki, Finland). The Ethics Committees of the Hospital for Children and Adolescents and the Department of Urology, University of Helsinki, approved the study protocol.

Tissue culture and treatments

Apoptosis of the human testicular germ cells was induced *in vitro* by incubating segments of seminiferous tubules under serum-free culture conditions. We cultured segments of seminiferous tubules, rather than isolated germ cells, in order to maintain the physiological contacts between the Sertoli cells and the germ cells. The testis tissue was microdissected on a Petri dish containing tissue culture medium (Nutrient mixture Ham's F10, Gibco Europe, Paisley, UK) supplemented with 0.1% human albumin (Sigma Chemicals Co, St. Louis, MO) and 10 µg/mL gentamicin (Gibco). Segments of seminiferous tubules (~ 2 mm in length) were isolated and transferred to culture plates containing the same tissue culture medium and cultured at 34°C in a humidified atmosphere containing 5% CO_2.

The effects of the following compounds on germ cell apoptosis were studied by adding them to the culture medium at the concentrations indicated: 17β-estradiol (10^{-10}, 10^{-9}, and 10^{-7} M; Sigma), ER antagonist ICI 182,780 (10^{-7} M; Tocris Cookson Ltd., Bristol, UK), 5α-androstan-17β-ol-3-one (DHT, 10^{-9}, 10^{-8}, and 10^{-7} M; Fluka Chemie Ag, Buchs, Switzerland), anti-FasL antibody sc-957/C-20 (1 and 20 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), caspase inhibitor Z-VAD.FMK (2 and 20 µM; Enzyme Systems, Dublin, CA), TNFα (1, 10, and 100 ng/ml; Roche Molecular Biochemicals, Mannheim, Germany or R&D Systems Ltd., Oxon, UK), sulfasalazine (SS, 5 mM; Fluka Chemie Ag, Buchs, Switzerland), acetyl salicylic acid (ASA, 5 mM; Sigma), N-acetyl-L-cysteine (NAC, 100 mM; Sigma), and NF-κB SN50 peptide (10 µg/ml; Biomol Research Laboratories, Plymouth Meeting, PA). Detailed descriptions of the use of these compounds are given in the original publications. In addition, the effects of sulindac (1 mM; Sigma) and indomethacin (25 µM; Sigma) were tested in experiments that were not included in the original publications.

Laboratory analyses

Southern blot analysis of DNA fragmentation

Genomic DNA was extracted from frozen segments of human seminiferous tubules, using the Apoptotic DNA Ladder Kit (Roche

Molecular Biochemicals, Mannheim, Germany), with some modifications. Briefly, the testis tissue samples were homogenized with an Ultra-Turrax T8 homogenizer and incubated for 10 min at room temperature in binding/lysis buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, and 20% TritonX-100, pH 4.4). The samples were then mixed with isopropanol (final conc. 25% v/v), loaded into polypropylene tubes, and centrifuged for 1 min at 8000 rpm. The tubes were washed twice with washing buffer (20 mM NaCl and 2 mM Tris-HCl, pH 7.5), and the bound DNA was eluted from the tubes with 10 mM Tris, pH 8.5. Finally, the samples were incubated with ribonuclease (2.5 µg/ml; deoxyribonuclease-free ribonuclease, Roche) for 20 min at room temperature. DNA was quantified spectrophotometrically (absorbance at 260 nm), and 1 µg of the DNA from each sample was subjected to 3´-end-labeling with digoxigenin-dideoxy-UTP (Dig-dd-UTP; Roche) by the terminal transferase (Roche) reaction. The DNA samples were then electrophoresed on 2% agarose gels, blotted onto nylon membranes, and crosslinked to the membranes by UV irradiation. The membranes were washed and blocked with 1% Blocking reagent (Roche) in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 30 min at room temperature. The 3´-end labeled DNA on the membranes was localized with alkaline phosphatase-conjugated anti-digoxigenin antibody (Anti-Digoxigenin-AP; Roche), and the bound antibody was detected by the chemiluminescence reaction (CSPD; Roche).

In situ end labeling (ISEL) of apoptotic DNA

Small segments of human seminiferous tubules (~1 mm in length) were squashed on microscope slides under coverslips to produce a monolayer of cells, and the preparations were fixed as previously described (176), with some modifications. Briefly, the slides were frozen in liquid nitrogen, after which the coverslips were removed and the frozen slides were dipped in ice-cold ethanol. The preparations were then fixed in 4% formalin for 10 min, washed in PBS, incubated in ice-cold ethanol:acetic acid (2:1) for 5 min, washed in distilled water, dehydrated, and stored at -20ºC. For ISEL, the squash preparations were first rehydrated, then washed in distilled water, and microwaved at high power for 5 min in citrate buffer (10 mM citrate, pH 6.0). After incubation for 10 min with terminal transferase reaction buffer (1 M potassium cacodylate, 125 mM Tris-HCl, and 1.25 mg/ml BSA, pH 6.6), the apoptotic DNA was 3´-end labeled with Dig-dd-UTP (Roche) for 1 h at 37°C by the terminal transferase reaction. For the negative controls, the terminal transferase enzyme was replaced with the same volume of distilled water. The preparations were then blocked with blocking solution (2% Blocking reagent, Roche, in 150 mM NaCl, 100 mM Tris-HCl, pH 7.5), followed by location of the Dig-dd-UTP with either alkaline phosphatase-conjugated (in the original publication II) or peroxidase-conjugated (in the original publications I, III, and IV) antidigoxigenin antibody (Anti-Digoxigenin-AP or Anti-Digoxigenin-POD, Roche). For detection of the antibodies, substrates for alkaline phosphatase (NBT, X-phosphate; Roche) or peroxidase (0.05% diaminobenzidine; Sigma) were added. Light counterstaining was performed with hematoxylin, after which the samples were dehydrated and mounted.

Electron microscopy

Segments of seminiferous tubules were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer,

dehydrated, and embedded in epoxy resin. The samples were then sectioned at 50 nm with a Reichert E Ultramicrotome (Reichert Jung, Vienna, Austria) and stained with uranyl acetate and lead citrate. The samples were examined with a Jeol JEM 1200 EX transmission electron microscope (Jeol, Tokyo, Japan).

Immunohistochemistry

Immunostainings of the ERα, ERβ, Fas, TNFR1, TNFR2, NF-κB RelA (p65), or NF-κB p50 proteins were performed on sections of formalin-fixed, paraffin-embedded adult human testis tissue or isolated seminiferous tubules, or on squash preparations of human seminiferous tubules. Paraffin sections were incubated at 60°C for 30 min and deparaffinized in xylene. Both paraffin sections and squash preparations were then rehydrated, microwaved at high power for 5 min in citrate buffer (10 mM citrate, pH 6.0) for antigen retrieval, washed, and blocked with blocking solution (PBS containing 5% goat normal serum, 3% BSA, and 0.1% Tween 20) for at least 30 min at room temperature. Our preliminary experiments revealed that unspecific staining for endogenous peroxidases only occurred in the erythrocytes of the testicular capillaries found in paraffin sections of testis tissue, and therefore, endogenous peroxidases were usually not blocked. The proteins under investigation were detected with the following antibodies: ERα HC-20 (sc-543, Santa Cruz), ERα NCL-ER-6F11 (Novocastra Laboratories Ltd., Newcastle, UK), ERα ER-1D5 (DAKO Corp. A/S, Glostrup, Denmark), ERβ PAI-313 (Affinity BioReagents, Inc., Golden, CO), Fas C-20 (sc-715, Santa Cruz), TNFR1 H-271 (sc-7895, Santa Cruz), TNFR1 CSA-810 (StressGen Biotechnologies Corp., Victoria, Canada), TNFR2 H-202 (sc-7862, Santa Cruz), NF-κB RelA (sc-109, Santa Cruz), or NF-κB p50 (sc-7178X, Santa Cruz). The optimal concentrations and incubation conditions for these antibodies are described in the original publications. The primary antibodies were detected using biotin-conjugated secondary IgGs from the corresponding ABC-Elite kits (Vector Laboratories, Inc., Burlingame, CA), followed by incubation with ABC solution. For location of the secondary antibody, 0.05% diaminobenzidine substrate (Sigma) was added. For the negative controls, the primary antibodies were replaced with nonspecific rabbit IgG (Sigma). Double immunostaining of the TNF-R1 and a macrophage surface antigen was performed, using the polyclonal TNFR1 H-271 antibody and a monoclonal antibody to the human macrophage surface antigen (HAM56, DAKO Corp. A/S, Glostrup, Denmark) as described in "Subjects and Methods" of III. After the staining protocols, light counterstaining was performed with hematoxylin, and the samples were dehydrated and mounted.

Protein extractions

For whole cell protein extracts, sections of testis tissue or segments of cultured seminiferous tubules were homogenized with an Ultra-Turrax T8 homogenizer into ice-cold homogenization buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 1 μg/ml leupeptin). The homogenates were vortexed vigorously, incubated on ice for 20 min, and centrifuged at 17,000*g* for 30 min. The resultant supernatants were used as whole cell protein extracts. For cytoplasmic and nuclear protein extracts, segments of seminiferous tubules were gently homogenized with a tight-fitting Potter-Elvehjem homogenizer into ice-cold hypotonic buffer (50 mM HEPES, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 0.2 mM

PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 0.5% Nonidet P-40), and cytoplasmic and nuclear protein extracts were prepared as previously described (177). Protein concentrations were determined by the Bradford method, using the Bio-Rad *DC* protein assay (Bio-Rad Laboratories, Hercules, CA). The protein extracts were stored in aliquots at -80°C until used for Western blotting or electrophoretic mobility shift assays.

Western blotting

Western blottings of the ERα, ERβ, Fas, FasL, TNFR1, TNFR2, and IκBα were performed on whole cell or cytoplasmic testicular protein extracts. Defined aliquots of proteins were loaded into SDS-polyacrylamide gels and electrophoresis was performed at 150 - 180 V. The proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corporation, Bedford, MA) and the transfer was checked by staining with 0.2% Ponceau S in 3% trichloroacetic acid. The proteins examined on the membranes were detected using the following antibodies: ERα HC-20 (sc-543, Santa Cruz), ERα H-184 (sc-7207, Santa Cruz), ERβ PAI-313 (Affinity BioReagents), Fas C-20 (sc-715, Santa Cruz), FasL (F37720, Transduction Laboratories, Lexington, KY), TNFR1 H-271 (sc-7895, Santa Cruz), TNFR1 CSA-810 (StressGen), TNFR2 H-202 (sc-7862, Santa Cruz), or IκBα (sc-371 and sc-847; Santa Cruz). The concentrations and incubation conditions for these antibodies are detailed in the original publications. When possible, the specificity of the bands detected with the antibodies was confirmed by preabsorption experiments. The primary antibodies were followed with peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) or peroxidase-conjugated goat anti-mouse IgG (DAKO Corp. A/S, Glostrup, Denmark). The bound secondary antibody was located with the ECL detection Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). After detection of the protein under investigation, the membrane was, on most occasions, washed and, as a loading control, probed with an antibody to α-tubulin (Sigma).

Electrophoretic mobility shift assay (EMSA)

A DNA probe containing a consensus κB enhancer element (5´-AGT TGA GGG GAC TTT CCC AGG C-3´) was purchased from Santa Cruz (sc-2505, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The probe was 5´-end-labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase (Promega Corporation, Madison, WI). Testicular nuclear protein extracts (10 μg) or control nuclear protein extracts (Jurkat T cells, Santa Cruz, sc-2132; KNRK cells, Santa Cruz, sc-2141; K562 cells, Santa Cruz, sc-2130; and LPS-stimulated human monocyte-derived macrophages, kindly provided by Dr Jukka Hakala, Wihuri Research Institute, Helsinki, Finland) (5-10 μg) were incubated on ice for 10 min with 2 μg poly(dI-dC)(dI-dC) (Amersham Pharmacia Biotech) in 50 mM HEPES (pH 7.6), 10% glycerol (vol./vol.), 225 mM KCl, 1 mM EDTA, 2.5 mM DTT, 1 mM $MgCl_2$, 0.75 mM PMSF and 1.5 μM leupeptin. A 5´-end-labeled probe (15 000 – 30 000 cpm) was then added, and incubation was continued at room temperature for 30 min. In the competition experiments, a 100-fold molar excess of unlabeled probe or unlabeled mutated probe (sc-2511, Santa Cruz) was added before the labeled probe. Reaction products were separated at room temperature on 4% polyacrylamide gels run in 22.5 mM Tris-borate, and 0.5 mM EDTA at 200 V. After

electrophoresis, the gels were dried and visualized by autoradiography. In the supershift assays, 2 μg of affinity-purified polyclonal antibodies were added after binding reactions, and incubation was further continued for 1 hour at room temperature. The antibodies for the supershift assays were purchased from Santa Cruz (RelA/p65, sc-109X; p50, sc-7178X; c-Rel, sc-272X; p52, sc-298X; RelB, sc-226X).

Quantitative analysis of x-ray films

The x-ray films exposed to chemiluminescense (Southern blots, Western blots) or autoradiography (EMSAs) were scanned with a tabletop scanner and the digital image was analyzed with the Gel plot 2 macro for the National Institutes of Health-Image 1.61 (Bethesda, MD, USA) (original publication II) or Scion Image beta 4.0.2 (Scion Corporation, Frederick, MD) (I, III, IV) image analysis software. For the Southern blots, the digitized quantification of the low-molecular-weight DNA fragments (< 1.3 kB) in the sample cultured for 4h or 5 h (or 10 h in time-course analysis of nuclear apoptosis; Figure 9, Figure 2 in IV) without treatments was taken as 1.0 (100% apoptosis), and the amounts of low-molecular-weight DNA fragments in the other samples were expressed in relation to this. For studies on alterations in the protein expression of the Fas and FasL (III), standard curves for Fas, FasL, and α-tubulin were generated with a dilution series of a control sample. The amounts of Fas or FasL in the samples were expressed in relation to the amount of α-tubulin in the corresponding samples. For time-course analysis of NF-κB activation (Figure 9, Figure 2 in IV), the digitized quantification of the specific NF-κB bands in the sample cultured for 5 h was set as 1.0, and the intensities of the bands in the other samples were expressed in relation to this.

Statistics

The experiments for Southern blot analysis of the effects of the tested compounds on apoptotic DNA fragmentation were repeated on at least three independent occasions. Quantitative data represent low molecular weight DNA (integrated optical density from scanned x-ray films). In the original publications I, III, and IV, data (mean ± SEM) were analyzed by one-way ANOVA, and when, significant differences were found, this was followed by comparison of the groups with a two-tailed unpaired Student's *t* test. $P < 0.05$ was considered statistically significant. Data demonstrating the time-courses of IκBα degradation, NF-κB DNA-binding activity, or nuclear apoptosis in IV are representative of two independent experiments. At least three independent experiments were conducted when alterations in the expressions of ERα, ERβ, Fas, or FasL were studied by Western blotting. At least three independent experiments were also conducted in which the effects of sulfasalazine, ASA, NAC, or SN-50 on NF-κB DNA binding activity were studied by EMSA or in which the effect of sulfasalazine on IκBα expression was studied by Western blotting. The effects of both sulindac and indomethacin were evaluated in two independent experiments. Each immunohistochemical picture or Western blot demonstrating the expression of a certain protein is representative of at least three independent experiments.

Results

In vitro induction of human male germ cell apoptosis

In the present *in vitro* model, human germ cells were cultured in their natural surroundings, *i.e.* in the seminiferous tubules, to maintain as physiological an environment as possible. Germ cell apoptosis was induced in this model by incubating segments of seminiferous tubules under serum-free culture conditions. Southern blot analysis of apoptotic DNA fragmentation in cultured segments of seminiferous tubules was used as the basic method for detecting testicular apoptosis. Apoptotic DNA fragmentation was observed within 4 to 5 hours and was further increased between 10 and 48 hours of culture. The time course of apoptotic DNA fragmentation from 0 to 10 hours is presented in Figure 5A.

In situ end-labeling (ISEL) of apoptotic DNA in squash preparations of uncultured and cultured

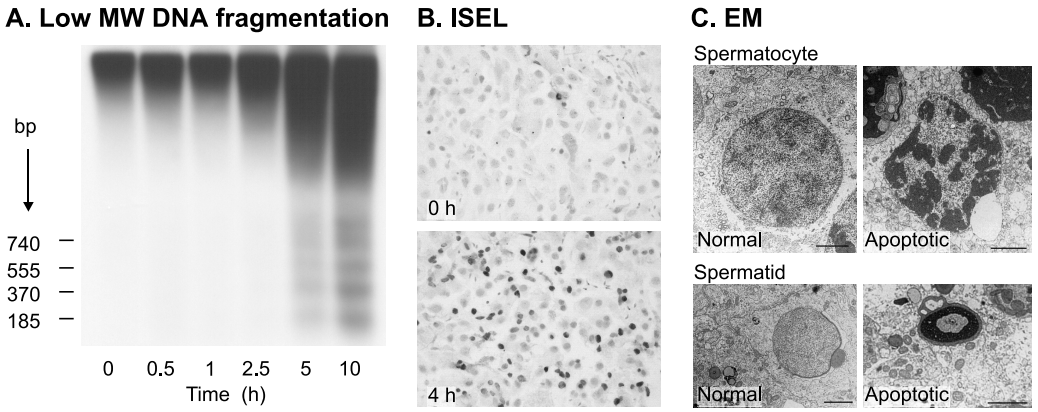

Fig. 5. *In vitro* induction of human male germ cell apoptosis. Segments of seminiferous tubules were cultured under serum-free conditions to induce germ cell apoptosis. **A.** Southern blot analysis of low-molecular-weight (MW) DNA fragmentation (185 bp multiples). DNA was extracted from the seminiferous tubules cultured for various periods of time, after which 1 µg of the total DNA in each sample was 3´-end labeled with Dig-dd-UTP, electrophoresed, and blotted onto a nylon membrane. The labeled DNA on the membrane was detected with chemiluminescence as described in "Materials and Methods". **B.** ISEL analysis of the induction of germ cell apoptosis. Uncultured (0 h) and cultured (4 h) segments of seminiferous tubules were squashed and fixed, and apoptotic DNA fragments were detected by 3´-end labeling with Dig-dd-UTP as described in "Materials and Methods". Very rare ISEL-positive cells (dark nuclei) were observed in uncultured tubule segments, whereas in samples cultured for 4 to 5 hours in apoptosis-inducing conditions, the number of ISEL-positive cells was greatly increased. Original magnification, x200. **C.** Electron micrographs of normal and late apoptotic pachytene spermatocytes and spermatids. For electron microscopy, segments of seminiferous tubules were fixed, sectioned at 70 nm, and stained as described in "Materials and Methods". Bars: 2 mm.

segments of seminiferous tubules (Figure 5B, Figure 4 in I, Figure 3 in II, Figure 2 in III, and Figure 4 in IV) was performed to confirm the results of Southern blotting, to identify the apoptotic cells, and to obtain information on the tissue morphology after culture. We used squash preparations rather than paraffin sections because (*i*) on account of the lack of false-positive labeling, which may occur after accidental formation of free DNA 3´-ends during permeabilization of paraffin sections, we considered the results of ISEL more reliable, and because (*ii*) in squash preparations, the nuclei of the cells maintain their characteristic morphology better, allowing more accurate identification of the individual cell types. Consistent with Southern blots, the number of ISEL-positive cells increased during culture. The cells undergoing apoptosis were found to be mainly premeiotic spermatocytes and postmeiotic spermatids.

The apoptotic nature of the cell death and the identification of the degenerating cells were further confirmed by electron microscopy (Figure 5C, Figure 1 in II), using the morphological criteria of apoptosis and the characteristic morphology of different germ cell types. Typical signs of the apoptotic cells were, for example, condensation of nuclear chromatin, degeneration of cytoplasmic organelles, and, in the later stages of apoptosis, dispersion of the nuclear envelope. The morphological signs of apoptosis were seen most often in spermatocytes and spermatids. Some of the apoptotic spermatids showed a ring-like condensation of chromatin around the nuclear periphery, which is characteristic of apoptosis in this type of germ cell. Late apoptotic cells were impossible to identify.

Effects of 17β-estradiol and dihydrotestosterone on male germ cell apoptosis

Inhibition of germ cell apoptosis by 17β-estradiol

To evaluate the effects of estrogens on male germ cells, we studied the role of the natural estrogen, 17β-estradiol, in germ cell apoptosis in the present culture model. Interestingly, 17β-estradiol effectively inhibited *in vitro*-induced germ cell death (Figure 6, Figure 3 in I). The most effective concentrations were 10^{-10} and 10^{-9} M, which are in the range of the previously reported physiological 17β-estradiol concentrations in human spermatic vein and testis tissue (178-181). In Southern blot analysis, the total amounts of low-molecular-weight DNA fragmentation were suppressed by 47% ($P < 0.001$) and 41% ($P < 0.01$) at 17β-estradiol concentrations of 10^{-10} and 10^{-9} M, respectively. A higher 17β-estradiol concentration of 10^{-7} M did not significantly inhibit germ cell death. Thus, it appears that 17β-estradiol prevents germ cell death at its physiological, but not higher, concentrations. Similarly, physiological, but not higher, concentrations of testosterone have previously been shown to prevent germ cell apoptosis in the present culture model (71). The suppressive effect of 17β-estradiol on germ cell apoptosis was blocked by the ER antagonist ICI 182,780 at 10^{-7} M (Figure 3 in I). ISEL of squash preparations from seminiferous tubules isolated immediately after orchidectomy (0 h) or cultured in serum-free conditions in the presence or absence of 17β-estradiol also indicated a clear inhibitory effect of 17β-estradiol on germ cell death (Figure 4 in I).

Expression of ERα and ERβ in the adult human seminiferous epithelium

Since the antiapoptotic action of 17β-estradiol may take place via activation of ERs, the locations of the ERα and ERβ proteins in the human seminiferous epithelium were studied in squash preparations of seminiferous tubules, using a rabbit polyclonal antibody (HC-20) and mouse monoclonal antibodies (ER-6F11 and ER-1D5) to human ERα and a rabbit polyclonal antibody to human ERβ. Strong positive staining for the ERα protein was observed in early meiotic germ cells (zygotene and early pachytene spermatocytes) and in early elongating spermatids (Figure 1 in I). Whether we used the polyclonal (HC-20) or the monoclonal (ER-6F11 or ER-1D5) antibodies to ERα, the result was the same (not shown). Of note, ERα immunoreactivity was located in the cytoplasm and/or the plasma membrane. The specificity of the staining with the HC-20 antibody was confirmed by preabsorption experiments with the corresponding peptide. The ERβ protein was found to be expressed by the same types of germ cells as the ERα (Figure 1 in I). ERβ immunoreactivity was found both in the nuclei and in the cytoplasm. Finally, Western blot analysis indicated the expression of both ERα and ERβ in the human seminiferous tubules (Figure 2 in I) and thus agreed with the results of immunohistochemistry.

Effect of DHT on *in vitro*-induced germ cell apoptosis

Testosterone, which inhibits germ cell apoptosis in the present model (71), can be metabolized *in vivo* to either 17β-estradiol or DHT. We therefore studied the effects of not only 17β-estradiol but also DHT on the *in vitro*-induced apoptotic death of testicular germ cells. DHT was also found to inhibit testicular apoptosis (Figure 6, Figure 5 in I). However, the lowest concentrations of DHT needed for effective inhibition of germ cell death were 100-1000 times the effective concentrations of 17β-estradiol, indicating the relatively high potency of

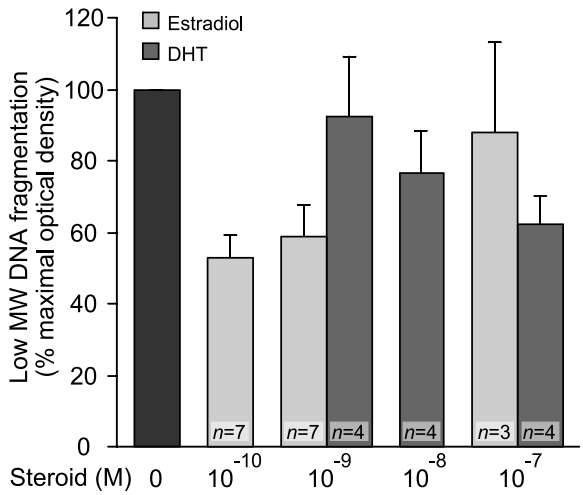

Fig. 6. Inhibition of male germ cell apoptosis by 17β-estradiol and DHT. Segments of seminiferous tubules were cultured for 4 hours under serum-free conditions in the absence or presence of 17β-estradiol or DHT. DNA was extracted from the seminiferous tubules and equal aliquots (1μg) of the total DNA in each sample were 3´-end labeled with Dig-dd-UTP. The aliquots were electrophoresed, subjected to Southern blotting, and analyzed with chemiluminescence, as described in "Materials and Methods". Low-molecular-weight (MW) DNA (<1.3 kB) fragmentation was quantified from x-ray films exposed to chemiluminescence. The digitized quantification of the low MW DNA fragments in the samples cultured in the absence of steroids was taken as 100% apoptosis, and the amounts of low MW DNA fragments in the other samples were expressed in relation to this. Each value represents the mean ± SEM of the indicated number of independent experiments.

estrogens as germ cell survival factors compared with that of androgens (Figure 6). Apoptotic low-molecular-weight DNA fragmentation was suppressed by 38% at a DHT concentration of 10^{-7} M ($P < 0.05$). Lower concentrations of DHT did not significantly inhibit germ cell death.

Regulation of germ cell apoptosis by the Fas system

Expression of Fas and FasL in the human testis

Localization of the Fas protein in the human seminiferous epithelium was studied immunohistochemically, using rabbit polyclonal antibody to human Fas. In squash preparations of uncultured segments of seminiferous tubules, strong positive staining for the Fas protein was found in spermatocytes and round spermatids, *i.e.* in the same cell types that underwent apoptosis in the cultured seminiferous tubules (Table 1, Figure 2 in II). The expressions of both Fas and FasL during germ cell apoptosis were then assessed by Western blot analysis of human seminiferous tubules cultured under serum-free conditions for increasing lengths of time. The expression of Fas remained constant at 0, 4, and 24 h (Figure 6 in II). In contrast, the expression of FasL was markedly up-regulated after 4 hours of culture (Figure 6 in III).

Inhibition of germ cell apoptosis by the antibody to FasL or by caspase inhibitor Z-VAD. FMK

To study the functional role of the Fas system in human germ cell apoptosis, we used the polyclonal anti-FasL antibody to inhibit apoptotic cell death in the culture of seminiferous tubules. This antibody binds to the carboxyl terminal part of the FasL, thus preventing interaction between Fas and FasL (182). Addition of this antibody to the culture caused clear inhibition of germ cell death when analyzed both by ISEL (Figure 3 in II) and by Southern blotting (Figure 7, Figure 4 in II). The anti-FasL antibody reduced the total amount of low-molecular-weight DNA fragmentation by 39% at a concentration of 20 μg/ml. Lower concentrations of the antibody were not effective. Because the downstream effectors of Fas include various caspases, we next tested the ability of a universal inhibitor of caspases, Z-VAD.FMK, to block germ cell apoptosis and found that it inhibited germ cell death (Figure 7, Figure 5 in II). DNA fragmentation was suppressed by 35% and 45% at the Z-VAD.FMK concentrations of 2 μM and 20 μM, respectively. Thus, in the present *in vitro*-model, Fas-FasL signaling appears to be involved in the initiation of germ cell apoptosis. Furthermore, this Fas-associated germ cell death is likely to be mediated by caspases.

Effects of TNFα on germ cell death and on the Fas system in cultured human seminiferous tubules

TNFR expression in the human testis

The presence of TNFR1 and TNFR2 in the human testis was first studied by Western blotting, and both receptors were detected (Figure 4 in III). However, by immunohistochemistry, specific staining was found only for TNFR1. The TNFR1 was detected

by two different antibodies in the Sertoli cells and interstitial Leydig cells (Table 1, Figure 5 in III). In the Sertoli cells, two kinds of staining were observed: *i*) a diffuse staining in the nuclei or in close proximity to the nuclei and *ii*) a spotted staining that was seen overall in the seminiferous epithelium and may represent cytosolic or plasma membrane staining of the Sertoli cells. Notably, no spotted staining was present in the neighboring peritubular myoid cells, which argues against the nonspecific nature of this staining pattern. In the immunoreactive interstitial cells, the staining was found in the cytoplasm. These cells were obviously Leydig cells, since they lacked the macrophage marker HAM56. Some clusters of TNFR1-expressing spermatogonia and pachytene spermatocytes were also found (Table 1), but these cells were far more rare than the immunoreactive Sertoli cells.

Inhibition of male germ cell apoptosis by TNFα

To evaluate the role of TNFα in germ cell apoptosis, we added recombinant human TNFα to the culture medium and studied its effects on the amount of apoptosis after culture for 4 hours in serum-free conditions. Interestingly, TNFα inhibited germ cell death effectively and dose-dependently (Figure 7, Figure 1 in III). In Southern blot analyses, the total amount of low-molecular-weight DNA fragmentation was suppressed by 25% ($P < 0.001$) and 43% ($P < 0.001$) at TNFα concentrations of 10 ng/ml and 100 ng/ml, respectively. A TNFα concentration of 1 ng/ml was also tested in three experiments, but it did not significantly inhibit germ cell death. ISEL of squash preparations from seminiferous tubules isolated immediately after orchidectomy (0 h) or cultured for 4 hours under serum-free conditions in the absence or presence of TNFα confirmed the results of Southern blotting (Figure 2 in III).

Because TNFα-induced survival is often associated with activation of the NF-κB pathway, we tested the ability of TNFα to increase the NF-κB DNA-binding activity in the present culture conditions. EMSAs of seminiferous tubules cultured for 0, 1, or 4 h under serum-free conditions indicated that NF-κB DNA-binding activity strongly increased during culture. However, TNFα did not affect this NF-κB activation (Figure 4 in III).

Down-regulation of the Fas ligand by TNFα

To study whether changes in the function of the Fas system could contribute to the observed anti-apoptotic effect of TNFα, the expressions of Fas and FasL were studied by Western blot analysis of seminiferous tubules cultured under serum-free conditions in the absence or presence of TNFα. We found that the expression of the Fas protein in the seminiferous tubules was not affected by TNFα (not shown). However, concomitantly with inhibiting

	Fas	TNFR1
Sertoli cells	-	+
Spermatogonia	-	(+)
Spermatocytes	+	(+)
Spermatids	+	-

Table 1. Expression of the death receptors Fas and TNFR1 in human seminiferous epithelium. The expression of Fas and TNFR1 was assessed by immunohistochemistry, using rabbit polyclonal antibodies to human Fas or TNFR1 as described in "Materials and Methods". Fas expression was studied in squash preparations of uncultured segments of seminiferous tubules and TNFR1 expression was studied in sections of formalin-fixed and paraffin-embedded uncultured human testis tissue.

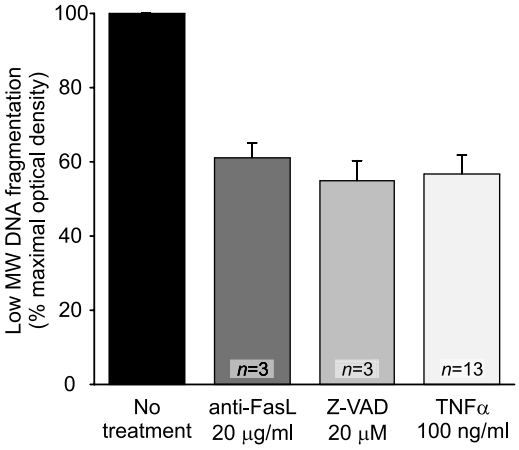

Fig. 7. Effects of modulation of the Fas- and TNFR1-mediated signaling pathways on male germ cell apoptosis *in vitro*. Segments of seminiferous tubules were cultured for 4 hours under serum-free conditions in the absence or presence of FasL blocking antibody C-20 (20 μg/ml), caspase inhibitor Z-VAD.FMK (20 μM), or recombinant human TNFα (100 ng/ml). Southern blot analysis of low-molecular-weight (MW) DNA (<1.3 kB) fragments in the tubule segments and quantification of the data were performed as described in "Materials and Methods". The digitized quantification of the low MW DNA fragments in the samples cultured in the absence of any treatment was taken as 100% apoptosis and the amount of low MW DNA fragments in samples treated with anti-FasL antibody, Z-VAD.FMK, or TNFα were expressed in relation to this. Each value represents the mean ± SEM of the indicated number of independent experiments.

testicular apoptosis, TNFα was found to regulate the expression of the FasL (Figure 8, Figure 6 in III). In five independent experiments, the expression of the FasL in tubules cultured under serum-free conditions for 4 hours was up-regulated by 48 – 185 % as compared with tubules that were not subjected to apoptosis-inducing conditions. TNFα decreased this up-regulated FasL expression by 14 - 72 %. The results were adjusted for the amount of α-tubulin present in the samples.

NF-κB activation in human testicular cell apoptosis

Constitutive NF-κB activity in the adult human testis

To explore the constitutive NF-κB activity in the adult human testis, the DNA binding activity of the κB-binding proteins was first studied by EMSAs, using nuclear protein extracts from uncultured human seminiferous tubules and a DNA probe containing a consensus κB-binding sequence. Three DNA-

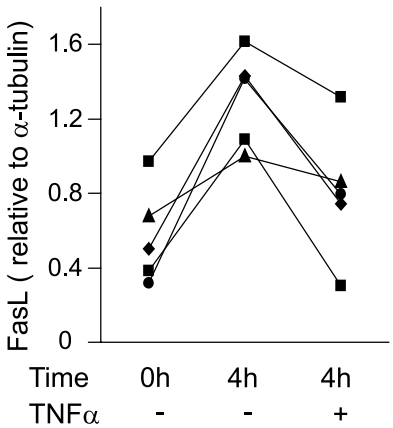

Fig. 8. Down-regulation of the testicular FasL by TNFα. Protein extracts from seminiferous tubules cultured for 0 and 4 hours under serum-free conditions in the absence or presence of 100 ng/ml TNFα were electrophoresed on 12% polyacrylamide gels, and analyzed by Western blotting as described in "Materials and Methods". FasL expression was quantified from Western blots and adjusted to the expression of α-tubulin, which was used as a loading control. In each experiment, the expression of the FasL was up-regulated in the tubules that were exposed to apoptosis-inducing conditions. This up-regulated expression of the FasL was decreased by TNFα.

protein complexes designated A, B, and C (Figure 1A in IV) were found. Band B was clearly seen, while bands A and C were poorly visible in the basal situation before exposure of the seminiferous tubules to apoptosis-inducing conditions. Competition experiments confirmed the specificity of the binding to the κB oligonucleotide (Figure 2 in IV). As demonstrated by the supershift assay, the NF-κB subunits RelA (p65) and p50 participated in the formation of the observed DNA-protein complexes (Figure 1A in IV).

Immunohistochemical studies using polyclonal antibodies to human RelA and p50 proteins were then conducted on sections of paraffin-embedded, formalin-fixed human testis tissue to determine the cell types expressing constitutively active NF-κB (Table 2, Figure 1B in IV). In the majority of the tubules, strong positive staining for RelA was found in the cytoplasm of some spermatogonia and early meiotic spermatocytes. In contrast, nuclear localization of the RelA, indicating expression of the active DNA binding protein, was observed in only a few scattered tubules. The cells showing positive nuclear immunostaining were identified as Sertoli cells. Because of the large volume of Sertoli cell cytoplasm and its partial rupture during sample preparation, direct comparison between Sertoli cell cytoplasmic and Sertoli cell nuclear or germ cell cytoplasmic staining is difficult. Thus, the light immunostaining that was observed around the Sertoli cell nuclei and germ cells most likely represents cytoplasmic expression of RelA in the Sertoli cells. A similar staining pattern was observed with an antibody against p50 (not shown). When the primary antibody was replaced with nonspecific rabbit IgG, no specific staining was found (negative control, shown in Figure 4 in IV).

Induction of testicular NF-κB during culture of human seminiferous tubules

The activation of NF-κB during testicular cell apoptosis was studied, using culture of human seminiferous tubules. Apoptotic DNA fragmentation was observed in cultured segments of seminiferous tubules within 5 hours and was further increased at 10 hours of culture (Figure 9, Figure 2A in IV). In the same tubules, NF-κB DNA binding activity had strongly and rapidly (within 30 min) increased from the basal level and remained at this elevated level throughout the culture for 10 hours (Figure 9, Figure 2B in IV). In EMSA, the intensity of band A especially was markedly strengthened during induction of testicular cell apoptosis. Western blot analysis of IκBα protein in the seminiferous tubules showed that, in agreement with the rapid increase in NF-κB DNA-binding activity, IκBα was readily degraded after withdrawal of serum (Figure 6A in IV). We therefore concluded that NF-κB activation occurs very early as compared with nuclear apoptosis (Figure 9, Figure 2C in IV).

EMSA supershift assays, using nuclear protein extracts from seminiferous tubules cultured for 2.5 hours in serum-free conditions, indicated that the inducible NF-κB complexes A and B are composed of the p50 and RelA subunits (Figure 3 in IV). Nuclear translocation of the p50 and RelA proteins was then studied immunohistochemically in sections of paraffin-embedded, formalin-fixed human seminiferous tubules. In all the segments of tubules cultured for 2.5 or 5 hours in serum-free conditions, nuclear translocation of RelA and p50 proteins

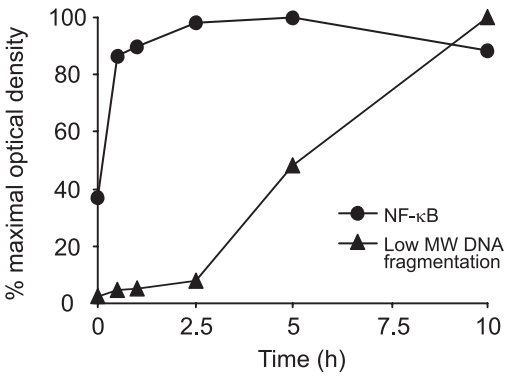

Fig. 9. Time-course of activation of the transcription factor NF-κB during germ cell apoptosis. Segments of seminiferous tubules were cultured under serum-free conditions for the indicated periods of time, after which apoptotic low-molecular-weight (MW) DNA (<1.3 kB) fragmentation was studied by Southern blotting and NF-κB DNA binding activity was studied by electrophoretic mobility shift assays (EMSAs) and quantified as described in "Materials and Methods". For Southern blots, the digitized quantification of the low MW DNA fragments in the sample cultured for 10 hours was taken as 100% apoptosis, and the amounts of low MW DNA fragments in the other samples were expressed in relation to this. For EMSAs, the digitized quantification of the specific NF-κB bands in the sample cultured for 5 hours was set as 100%, and the intensities of the bands in the other samples were expressed in relation to this. The results shown are representative of two independent experiments.

was observed in Sertoli cells, indicating that the site of NF-κB activation was the Sertoli cells (Table 2). Of note, nuclear translocation of the NF-κB proteins was not observed in the spermatogonia or in the early meiotic spermatocytes, in which cytoplasmic staining for RelA and p50 was intense in the 0 h samples (Table 2). Since the presence and the nuclear translocation of RelA and p50 were similar, only the RelA immunostaining is shown in Figure 4 of IV.

When considering the potential role of NF-κB in the regulation of apoptosis in the testis, it was of interest to determine whether NF-κB activation occurred in the cell types undergoing apoptosis. Therefore, we performed RelA immunostainining and ISEL of squash preparations of human seminiferous tubules that had not been exposed to apoptosis-inducing conditions, or that had been cultured in serum-free conditions to induce apoptotic cell death

	Constitutive cytoplasmic NF-κB	Constitutive nuclear NF-κB	Inducible nuclear NF-κB	ISEL staining
Sertoli cells	+	+	+++	-
Spermatogonia	+	-	-	(+)
Spermatocytes	+	-	-	+++
Spermatids	-	-	-	+++

Table 2. Cellular localization of NF-κB immunostaining and ISEL positivity (apoptotic DNA fragments). Constitutive NF-κB expression in human seminiferous epithelium was assessed by immunostaining the RelA and p50 NF-κB subunits in paraffin-embedded, formalin-fixed sections of testis tissue or in squash preparations of uncultured segments of seminiferous tubules, using polyclonal antibodies to the corresponding human proteins, as described in "Materials and Methods". Cellular localization of the inducible nuclear NF-κB expression and ISEL positivity were studied in formalin-fixed, paraffin-embedded sections or squash preparations of segments of seminiferous tubules cultured for 2.5 and 5 h under serum-free conditions. Nuclear expression of the NF-κB proteins usually indicates that they are active. Thus, NF-κB activation appears to occur in Sertoli cells, whereas apoptotic DNA fragmentation takes place predominantly in spermatocytes and spermatids.

(Table 2, Figure 4B in IV). In agreement with the results in the paraffin sections, nuclear translocation of RelA was observed in the Sertoli cells. In contrast, in the ISEL analysis, apoptotic DNA fragmentation was predominantly observed in the late meiotic and postmeiotic germ cells, whereas the Sertoli cell nuclei remained negative. Thus, NF-κB activation was induced in the Sertoli cells, while apoptotic cell death occurred in germ cells.

Effects of anti-inflammatory and NF-κB inhibitory compounds on stress-induced male germ cell apoptosis

Fas activation, TNFα-mediated signaling, and NF-κB-induced gene expression have all been found to be associated with immune and inflammatory responses. Therefore, we tested the ability of previously reported anti-inflammatory and NF-κB inhibitory compounds to modulate apoptotic germ cell death in our *in vitro* model.

Sulfasalazine

Both germ cell apoptosis and NF-κB induction were effectively blocked by the anti-inflammatory drug sulfasalazine (Table 3, Figure 5 in IV). During 5 hours of culture, a 5 mM concentration of sulfasalazine (SS) inhibited apoptotic DNA fragmentation by 87% ($P < 0.001$) and concomitantly retained NF-κB DNA binding activity at the basal (0 h) level after 2.5 hours and 5 hours. In two independent experiments, effective inhibition of apoptotic DNA fragmentation by sulfasalazine was still observed after 48 hours of culture (not shown). Suppression of germ cell death was also observed in ISEL of seminiferous tubules cultured in the absence or presence of sulfasalazine. After the treatment, no severe abnormalities were seen in the morphology of the cells of the seminiferous epithelium.

As sulfasalazine has previously been reported to inhibit NF-κB activity in certain cell types by inhibiting IκB degradation (183,184), we wished to know whether the same inhibitory mechanism would function in the seminiferous epithelium. Although Western blot analysis of cultured seminiferous tubules showed that the IκBα protein was degraded, it did not completely disappear; the lowest level was observed at 2.5 h, after which the expression of IκBα gradually increased slightly (Figure 6A in IV). This is likely to be explained by the known ability of activated NF-κB to stimulate transcription of the IκBα gene. IκBα degradation was not prevented in the samples treated with 5 mM sulfasalazine (Figure 6A in IV), suggesting that sulfasalazine inhibits NF-κB activation at a level distal to that of IκB degradation. In contrast, in sulfasalazine-treated tubules after 5 hours of culture, Western blotting revealed the complete disappearance of the IκBα protein, suggesting that sulfasalazine prevented the NF-κB-induced synthesis of new IκBα protein. In agreement with the results of the IκBα Western blots, RelA immunostaining of sulfasalazine-treated seminiferous tubules showed that nuclear translocation of the RelA protein in the Sertoli cells was not prevented by sulfasalazine (Figure 6B in IV). Thus, in this apoptosis model, sulfasalazine seemed to inhibit NF-κB DNA binding in the nucleus.

Finally, as the promoter of the gene encoding FasL is known to contain NF-κB binding sites

(171,185), and as the regulation of this gene has been suggested to be a potential target for NF-κB in the testis (175), we tested the effect of the sulfasalazine-mediated NF-κB blockade on the expression of the FasL protein in Western blot analysis. Despite the effective inhibition of NF-κB activation, sulfasalazine had no effect on the protein expression of the FasL after culture for 2.5 h and 5 h (not shown), suggesting that NF-κB does not regulate testicular FasL expression during stress-induced apoptosis.

Other compounds

A NF-κB inhibitory peptide SN-50 (10 μg/mL), which interferes with the nuclear translocation of activated NF-κB dimers, slightly inhibited testicular apoptosis (24%, $P = 0.01$), but an inhibitory effect of this peptide on NF-κB activation could not be reliably detected with EMSAs (Table 3). Other previously reported NF-κB inhibitors, acetyl salicylic acid (ASA) and N-acetyl-L-cysteine (NAC), were found to be effective inhibitors of germ cell apoptosis (Table 3, Figure 5A in IV). Apoptotic DNA fragmentation was reduced by 44% ($P < 0.05$) with ASA at 5 mM and by 87% ($P = 0.001$) with NAC at 100 mM. ISEL analysis confirmed the results of Southern blotting and showed that the overall morphology of the testicular cells was normal after the treatments with ASA and with NAC (Figure 5C in IV). However, although capable of inhibiting apoptosis, ASA and NAC had no effect on NF-κB DNA binding (Table 3, Figure 5B in IV). In addition, the effects on testicular NF-κB DNA binding and apoptosis of two other anti-inflammatory drugs, sulindac (1 mM), which inhibits NF-κB in some cell lines (186), and indomethasin (25 μM), which should not affect NF-κB (187), were tested in two independent experiments. Both compounds inhibited apoptotic cell death, but did not affect NF-κB DNA binding (Table 3). Thus, the anti-apoptotic effects of ASA, NAC, sulindac, and indomethasin appear to be mediated by mechanisms other than NF-κB inhibition.

	NF-κB DNA binding activity	Germ cell apoptosis
Sulfasalazine	↓↓↓	↓↓↓
SN-50 peptide	(↓)	↓
Acetyl salicylic acid	↔	↓↓
N-acetyl-L-cysteine	↔	↓↓↓
Sulindac	↔	↓↓
Indomethacin	↔	↓

Table 3. Effects of anti-inflammatory and NF-κB inhibitory compounds on seminiferous tubule NF-kB DNA-binding activity and germ cell apoptosis. Segments of seminiferous tubules were cultured under serum-free conditions in the absence or presence of 5 mM sulfasalazine, 5 mM acetyl salicylic acid, 100 mM N-acetyl-L-cysteine, 10 μg/mL NF-kB SN50 peptide, 1 mM sulindac, or 25 μM indomethacin. After culture for 2.5 hours, samples of the tubules were snap-frozen and the nuclear proteins were prepared for analysis of NF-κB activity. The rest of the tubules were cultured for 5 hours and analyzed for apoptotic DNA fragmentation. NF-κB DNA binding activity was analyzed by EMSAs and apoptotic DNA fragmentation by Southern blotting, as described in "Materials and Methods".

Discussion

The present study aimed at elucidating the regulation of human testicular apoptosis, using cultured human seminiferous tubules as a model of apoptosis. In this model, the steroid hormones 17β-estradiol and DHT at their physiological concentrations were found to suppress germ cell death. Unexpectedly, 17β-estradiol appeared to be an even more potent inhibitor of testicular apoptosis than the non-aromatizable androgen DHT. The studies were then focused on the signaling pathways initiated by FasL and by TNFα, the cytokines known as "death factors". It was found that *i*) human testicular germ cell apoptosis is mediated by the Fas system, *ii*) TNFα protects male germ cells from apoptotic death and opposes the Fas system by down-regulating the FasL, and *iii*) the transcription factor NF-κB, which is often considered to be a mediator of TNFα-induced survival signals, appears not to mediate the antiapoptotic effect of TNFα, but to function in parallel with or downstream of the Fas pathway to regulate human testicular germ cell apoptosis. Finally, as the Fas and TNF systems, as well as NF-κB, are mediators of human inflammatory responses, the ability of anti-inflammatory compounds to inhibit testicular apoptosis was studied. Interestingly, common anti-inflammatory drugs were found to effectively inhibit germ cell apoptosis, raising the possibility that they could be used therapeutically in transient stress situations involving excessive germ cell death and consequent male infertility.

Methodological aspects

In the present study, we used an *in vitro* tissue culture for investigating the regulation of human testicular cell apoptosis. Our culture model, like all *in vitro* models, has limitations. Activation of specific apoptotic pathways depends on the type of stressor, and the culture of seminiferous tubules under serum-free conditions involves not only deprivation of serum and growth-factors, but also relative hyperoxia due to the high partial oxygen pressure in the normal atmosphere as compared with that found in peripheral organs such as the testis. Thus, the apoptotic pathways induced in the present model are potentially multiple and there may also be multiple inducers of a specific pathway. Moreover, various stimuli may differentially activate certain pathways in different types of testicular cells. Therefore, mechanistic and inhibitory studies of specific apoptotic pathways are difficult to conduct in the present model. It would be interesting to study the Fas system, TNFα-mediated signaling, or NF-κB activity following either specific Sertoli cell toxicants (66,69) or disturbers of germ cells such as radiation (79,188). However, the culture conditions are difficult to optimize so that no germ cell apoptosis occurs, which is a prerequisite for the conduction of such experiments. Culturing isolated cells, in turn, is inappropriate, because interactions between different types of seminiferous epithelial cells play an important role in testicular physiology and pathology. In support of this, maturation of isolated germ cells from spermatogonial stem cells to the meiotic prophase has not been

achieved in any published study *in vitro* (2). It appears that both the initiation of spermatogonial differentiation from stem cells and the entry of differentiating spermatogonia into meiosis are blocked in a culture of isolated cells. Moreover, Sertoli cells have been shown to inhibit apoptosis of spermatocytes and round spermatids in *in vitro* culture conditions (189), supporting the need of a culture model that maintains cell-to-cell contacts when studying the regulation of germ cell apoptosis. Furthermore, *in vivo* studies with apoptosis-modulating treatments are virtually impossible in humans. Finally, because of the potential species specificity of cellular responses, results obtained in animal studies do not necessarily apply to humans. Therefore, we feel that, despite its limitations, the present *in vitro* model, which maintains physiological contacts between the cells of the seminiferous epithelium, affords the best available way to evaluate human testicular apoptotic mechanisms involving interactions between different cell types. The present culture system models a situation in which human testicular homeostasis is threatened, demonstrates how different types of cells in the seminiferous epithelium may act during severe stress, and affords an opportunity to study the effects of pharmacological modulation of stress-induced apoptosis in the human testis.

For our experiments, we used testis tissue samples from men undergoing orchidectomy as treatment for prostate cancer. The testis tissue in these patients was normal and was removed in order to stop physiological production of hormones that stimulate the growth of the cancer cells. Despite the old age of most of these patients, germ cells at all phases of maturation were found in light microscopic and electron microscopic evaluation of the tissue. Samples from younger men were only available as testicular biopsies that were taken because of infertility or suspected disease or as testis tissue that was removed because of testicular cancer. In all these cases, the samples were either too small for our experiments or were disturbed by disease. Therefore, we considered the orchidectomy samples appropriate and the best available samples for the present studies on human testicular apoptosis.

17β-estradiol as a survival factor for male germ cells

Estrogens have recently been shown to be essential for male reproduction (97,99,190), but the exact roles of estrogen in spermatogenesis have remained unclear. In the present study, we demonstrate that, in addition to the previously suggested indirect effects of estrogens on the seminiferous epithelium, estrogens may contribute directly to the survival of germ cells in the testis. Interestingly, we found that the *in vitro*-induced apoptosis of male germ cells was effectively inhibited when 17β-estradiol was added to the culture medium. As only seminiferous tubules and some occasional interstitial Leydig cells, but not other parts of the testis, were present in our culture model, this action of 17β-estradiol must be a direct effect either on the cells of the seminiferous tubules or on the Leydig cells. Although estrogens have a well-documented inhibitory effect on Leydig cell androgen secretion and RNA synthesis, the concentrations of 17β-estradiol needed for *in vitro* inhibition of Leydig cell function in the previous studies (191-194) were at least 1000-fold the concentrations capable of effectively inhibiting germ cell apoptosis in the present study. Therefore, the possible inhibitory

effect of these low apoptosis-inhibiting concentrations of 17β-estradiol on the occasional Leydig cells present in our culture model and consequent indirect effects of 17β-estradiol on germ cell survival seem unlikely.

The suppressive effect of 17β-estradiol on germ cell apoptosis was blocked by an ER antagonist, ICI 182,780, suggesting that the effects of 17β-estradiol were mediated by some types of ERs. The classic ER signaling pathway involves binding of the ligand-bound ER to the estrogen-responsive element that regulates transcription of target genes in the nucleus. ERs may also regulate gene transcription by binding to proteins within a preformed transcriptional complex, such as the AP1 transcription factor complex (92,195). In addition to the regulation of gene transcription by ligand-bound nuclear ERs (nERs), recent studies have revealed evidence for the existence of functional plasma membrane estrogen receptors (pmERs) (196-198). These receptors may share structural similarities with classical nERs, for a variety of antibodies directed against multiple epitopes of the nuclear ERα identify a membrane protein in several cell types (197). In response to 17β-estradiol, pmERs induce rapid signaling events such as activation of phosphoinositol 3-kinase, protein kinase B/Akt, and ERK and p38 MAP kinases. Moreover, it is possible that the large cytosolic pool of ERs, envisioned as a reservoir before it moves to the nucleus, can in fact act on cytosolic organelles. Indeed, it has been suggested that, as an anti-apoptotic factor, cytosolic estradiol-ER complexes may locally regulate mitochondrial membrane potential (197,199). Thus, it appears that 17β-estradiol can interact with distinct, compartmentalized pools of receptors, each having unique effects on cellular physiology and possibly also on cell survival. As in the present study the antiapoptotic effect of 17β-estradiol on germ cells was seen after incubation for 4 h, it is possible that the survival of germ cells is mediated at least partly by rapid signaling pathways induced by the non-nuclear ERs.

Our finding that both the ERα and ERβ proteins are expressed in early meiotic and postmeiotic germ cells of the human testis suggests direct effects of 17β-estradiol on these cells. Antibodies against ERα protein reacted with a cytoplasmic and/or plasma membrane protein, which supports a role for extranuclear (plasma membrane or cytosolic) ERs in 17β-estradiol-mediated germ cell survival. In the cells staining positively for ERβ, immunoreactivity was found both in the nuclei and in the cytoplasm. The presence of ERβ protein in testicular germ cells is in accord with the previously reported expression pattern of ERβ mRNA in the human testis (200). The presence of ERα, however, has been found only in rat spermatocytes and spermatids in one study (201), whereas in other studies on testicular ER expression, neither ERα mRNA nor protein has been detected in germ cells (92). In our study, we confirmed the results obtained with the polyclonal ERα antibody with two widely used and well-characterized monoclonal antibodies to human ERα (202). Moreover, we followed standard immunohistochemical protocols. Therefore, the discrepancy between the results is most likely caused by differences in the samples used in immunohistochemistry. We detected the ERs in squash preparations of human seminiferous tubules, which have not been used in any of the other studies. We decided to use squash preparations because the literature on ER expression in samples of other types is highly controversial (92), and because in the squash

preparations the cells of the seminiferous epithelium maintain their morphology better than in frozen or paraffin sections of the human testis. Since our results obtained with these preparations differ from the results of most previous studies, the human testicular expression of the ERα or an extranuclear ERα-like ER remains to be confirmed. If only ERβ, and no ERα or other ERs, is expressed in testicular germ cells and possibly also in Sertoli cells (92), the lack of any spermatogenic disruptions in ERβKO mice is surprising. Interestingly, a recent study showed that testicular germ cells from ERαKO mice could develop normally when transplanted into wild-type mice, suggesting that germ cells do not require ERα expression for development and function (203). Accordingly, the roles of germ cell ERα, ERβ, and other types of ER as mediators of the anti-apoptotic effect of 17β-estradiol remains to be clarified. Moreover, the possible contribution of Sertoli cells to 17β-estradiol-mediated germ cell survival remains unknown.

In the previous experiments conducted by our study group, testosterone was shown to be an effective inhibitor of germ cell apoptosis in the human testis *in vitro* (71). However, the concentrations of testosterone (10^{-7} M) required for this apoptosis-inhibiting effect were 100-1000 times the effective concentrations of 17β-estradiol (10^{-9}-10^{-10} M). Of note, the effective concentrations of testosterone and 17β-estradiol are in the range of their relative physiological levels in the spermatic vein (178,179,181,204,205) and in testis tissue (180,206). *In vivo*, testosterone can be metabolized to either estrogens or DHT. We found that DHT was also capable of inhibiting germ cell death in our *in vitro* model, but, like testosterone, its lowest effective concentrations were strikingly higher than the effective concentrations of 17β-estradiol. Thus, *in vitro* 17β-estradiol appears to be a more potent inhibitor of male germ cell death than the androgens testosterone and DHT. The essential role of androgens in completing normal spermatogenesis is well established. However, the mechanism by which androgens regulate spermatogenesis has not been resolved, and even the site of androgen action within the testis has remained unclear. Some reports have shown an immunoreactive androgen receptor (AR) in developing germ cells (207-210), but most suggest that only testicular somatic cells contain AR (89,211-214). If the germ cells lack functional AR, the effects of androgens on spermatogenesis may be mediated by the somatic cells. The results of the present study suggest another interesting mechanism for testosterone-mediated survival of germ cells. Taken the present result that 17β-estradiol is much more potent than androgens in inhibiting germ cell apoptosis and the previously reported expression of P450 aromatase by adult Leydig and germ cells in several species (93-95,215-217), it is possible that, to mediate its protective effects on germ cells, testosterone is at least to some extent metabolized to estrogen. This hypothesis is supported by the results from mice lacking aromatase (ArKO), which were found to have increased germ cell apoptosis in the adult testis (99). The AR-mediated action of androgens, in turn, is needed for normal development of male reproductive organs, as indicated by the phenotype of human 46,XY individuals with the androgen insensitivity syndrome, which is caused by the absence or abnormality of the AR (218-220). In complete androgen resistance, the patients have a female body habitus, female external genitalia, and bilateral, undescended testes with abnormal

morphology and progressive reduction in the number of germ cells with increasing age. Thus, AR-mediated androgen effects appear to be indispensable for testicular development, whereas estrogens are possibly needed for the testosterone-mediated survival of germ cells in the adult testis.

Taken together, our results strongly suggest that estrogens act as germ cell survival factors in the human testis. This effect of estrogens may be mediated through conventional ERs, but signaling by a nonclassical ER or via a nongenomic pathway is also possible. The present results, together with the results of studies on ERαKO (97,98) and ArKO (99) mice, indicate the importance of estrogens for the normal function of the adult testis.

Fas- and TNFR1-mediated signaling in the regulation of human male germ cell death

The closely related signaling pathways initiated by the cytokines FasL and TNFα were of interest to us, because they are known to mediate apoptosis in many cell types and they have both been suggested to play a role in the regulation of spermatogenesis. Fas had previously been shown to induce apoptosis in the rodent testis, but human studies were not available. TNFα had been shown to be secreted by testicular germ cells and to have some effects on cultured Sertoli and Leydig cells, but the effects of this cytokine on maturing germ cells was unknown and its role in the regulation of spermatogenesis was unclear.

In the present study, the Fas system was found to mediate germ cell apoptosis in cultured human seminiferous tubules (Figure 10). Fas was expressed in pachytene spermatocytes and round spermatids, which are the same types of cells that underwent apoptosis in the present culture model. Moreover, germ cell death was inhibited by blocking the interaction between Fas and FasL. These results are in agreement with the rodent data showing that Fas activation plays a role in mouse and rat testicular apoptosis (140,142). However, although Fas is up-regulated after many treatments that induce massive germ cell apoptosis in the rodent testis (140,142), we did not observe increased expression of the Fas protein during culture of the seminiferous tubules. In contrast, up-regulation of testicular FasL was associated with germ cell apoptosis suggesting that, in cultured human seminiferous tubules, increased germ cell death is at least partly caused by elevated levels of FasL. In the rodent testis, it appears that injury of germ cells results in up-regulated Fas, whereas injury of Sertoli cells triggers increased production of FasL (142). In the present study, however, the Sertoli cells were usually not injured and survived. Thus, the results of rodent studies do not seem to apply to the human germ cell apoptosis induced in the present culture model. This may be explained by the different apoptosis models or may indicate species specificity in the regulation of the Fas system.

In contrast to FasL, TNFα was found to suppress testicular germ cell death (Figure 10). This effect of TNFα appears to result from TNFR1 activation, because only TNFR1 was found by immunohistochemistry in the uncultured human testis. Within the seminiferous epithelium, occasional germ cells stained positively for TNFR1, which may

indicate a direct effect of TNFα in these cells. However, the most abundant expression of the TNFRI was seen in the Sertoli cells. In these cells, two kinds of staining were observed; a diffuse staining located more or less in the nucleus and a spotted staining located in the cytoplasm or the plasma membrane. Although TNFR1 is traditionally regarded as a cell surface receptor, recent reports have shown that the Golgi apparatus is a major site of TNFR1 expression, and in some cell types, such as endothelial cells, TNFR1 is primarily a Golgi-associated protein, only a minor subpopulation of receptors being present in the plasma membrane (221-223). The Golgi-associated TNFR1 has been shown to have a perinuclear immunostaining pattern. Moreover, phosphorylation of the TNFR1 by extracellular signal-regulated kinase $p42^{mapk/erk2}$ leads to redistribution of the phosphorylated receptor from the Golgi apparatus and plasma membrane to perinuclear tubular structures that are associated with the endoplasmic reticulum (221). Thus, the nuclear TNFR1 staining pattern found in the human Sertoli cells may represent TNFR1 in either the Golgi apparatus or the endoplasmic reticulum-associated tubular structures. The spotted staining, in turn, is likely to represent a receptor population located in the Sertoli cell plasma membrane, since in the plasma membrane, TNFR1 is associated with lipid rafts, which have been shown to exhibit this kind of staining pattern (224). The physiological significance of the alterations in the subcellular localization of the TNFR1 is unclear, although they apparently lead to changes in the signaling properties of the receptor (224,225). Thus, in the endoplasmic reticulum, phosphorylated TNFR1 recruits Bcl-2 and phosphorylation-induced redistribution of the TNFR1 protects against apoptosis by a Bcl-2-dependent mechanism (225). It is also possible that the Golgi apparatus, known to be strongly enriched in unesterified cholesterol, a major component of lipid rafts, is a storage site for TNFR1, from which the receptor can be translocated to the lipid rafts on the plasma membrane upon appropriate stimuli (224).

The predominant expression of the TNFR1 in the Sertoli cells suggests a paracrine mechanism for the TNFα-induced survival of germ cells. Interestingly, suppression of germ cell death by TNFα was associated with decreased testicular expression of FasL. Down-regulation of the death-promoting FasL may therefore mediate the survival signal induced by TNFα (Figure 10). A similar mechanism of TNFα-mediated inhibition of apoptosis was observed in the vascular endothelium, where down-regulation of FasL expression in the endothelial cells by TNFα led to decreased endothelial cytotoxicity toward Fas-bearing leukocytes (226). However, the mechanism by which TNFα-induced signaling may affect the expression of FasL remains unclear. In cell types resistant to pro-apoptotic signals of TNFα, the survival signals induced by this cytokine are often associated with activation of the NF-κB pathway (40,135,137). Moreover, the promoter of the gene encoding FasL is known to contain NF-κB binding sites (171,185), suggesting that NF-κB is a potential regulator of FasL. In the present study, NF-κB DNA binding activity was increased in seminiferous tubules cultured under serum-free conditions and showing increased apoptosis. However, TNFα appeared to have no effect on this NF-κB activation, suggesting that, in the human testis, TNFα may induce down-regulation of the testicular FasL and germ cell survival via a pathway distinct from NF-κB activation.

NF-κB in male germ cell apoptosis

NF-κB is an interesting candidate for regulation of male germ cell apoptosis, for there is growing evidence that this transcription factor has a function in cell proliferation and apoptosis, and recent studies suggest a role for NF-κB in mammalian spermatogenesis. Moreover, the surprising finding that NF-κB was strongly activated during *in vitro*-induced testicular apoptosis, but that this activation did not appear to be associated with the TNFα-induced germ cell survival, prompted us to undertake a further investigation of NF-κB activity in the normal human testis and its role in testis tissue undergoing apoptosis.

Consistently with the results for the rat testis (174), we found low basal NF-κB DNA binding activity, which, by immunostaining of the RelA and p50 subunits, was localized to the Sertoli cells. However, in contrast to the rat testis, in which Sertoli cells express nuclear NF-κB at all stages of spermatogenesis (174), in the human testis only a few segments of the seminiferous tubules contained Sertoli cell nuclei that were immunoreactive. Moreover, we found no germ cells showing nuclear NF-κB, which contrasts with the previously observed stage-dependent expression of nuclear NF-κB in the late meiotic and postmeiotic germ cells of the rat testis (174). However, intense cytoplasmic staining for NF-κB was found in the spermatogonia and early meiotic germ cells of the human testis. As no nuclear translocation of this cytoplasmic NF-κB was observed in cultured seminiferous tubules, the physiological significance of this finding remains obscure.

During *in vitro*-induced testicular apoptosis, the Sertoli cell nuclear NF-κB expression and the whole seminiferous tubule NF-κB DNA binding activity increased strongly before the onset of nuclear apoptosis. Thus, it was of interest that apoptosis was induced in late meiotic and postmeiotic germ cells, while the Sertoli cells containing the inducible NF-κB survived. NF-κB activation of the Sertoli cells may therefore serve to protect the Sertoli cells themselves and, simultaneously, to induce Sertoli cell production of factors that are able to mediate germ cell death. This would be a reasonable way for the Sertoli cells to act, since they are essential for functional spermatogenesis and they are terminally differentiated cells with no capacity for renewal (8). Interestingly, induction of testicular NF-κB DNA binding activity was completely blocked by the anti-inflammatory aminosalicylate drug sulfasalazine, which has also been shown to specifically inhibit NF-κB activation in colon epithelial cells and in Jurkat T cells (183). Concomitantly, germ cell apoptosis was effectively suppressed, supporting the hypothesized role for NF-κB as one of the factors controlling stress-induced germ cell death. However, the Sertoli cells survived despite sulfasalazine treatment, suggesting that Sertoli cell survival does not depend on their activation of NF-κB.

Sulfasalazine has previously been reported to inhibit NF-κB activation by directly inhibiting the IκB kinases α and β, which leads to inhibition of IκBα degradation (184). In the present study, sulfasalazine inhibited neither IκBα degradation nor nuclear translocation of the RelA subunit, but prevented the formation of new IκBα protein, thus resulting in the absence of IκBα from sulfasalazine-treated seminiferous tubules. This most likely indicates

a decrease in NF-κB mediated transcriptional activation of the IκBα gene. Interestingly, the cytoplasmic NF-κB in some immature germ cells did not translocate into the nucleus, despite the disappearance of the IκBα. This may indicate that the type of IκB in these cells is other than IκBα. Indeed, high levels of IκBβ and IκBε mRNA have been found in the testis (227,228), which suggests that these forms of IκB participate in the regulation of testicular NF-κB.

Finally, we wished to find out whether the testicular expression of FasL is regulated by NF-κB, because this cytokine plays an important role in the control of germ cell apoptosis (140,142), and because, in certain forms of apoptosis, its expression has been shown to be directly regulated by NF-κB (170,171). The expression of the FasL is up-regulated during culture of the seminiferous tubules. If this was due to NF-κB dependent transcription of the gene encoding the FasL, then sulfasalazine, which effectively blocks NF-κB DNA binding activity, should affect the expression of the FasL. However, we found no effect of sulfasalazine on the levels of the FasL, suggesting that NF-κB does not regulate testicular FasL gene expression during *in vitro*-induced germ cell apoptosis. This agrees with the result showing that TNFα-mediated inhibition of germ cell apoptosis is associated with down-regulation of the FasL, but not with modulation of the inducible NF-κB activity. Thus, it can be suggested that in a testicular stress situation caused by external disturbances, the NF-κB- and FasL-mediated pathways would function in parallel to regulate human testicular germ cell death, or the final action of NF-κB would take place downstream of the action of FasL (Figure 10).

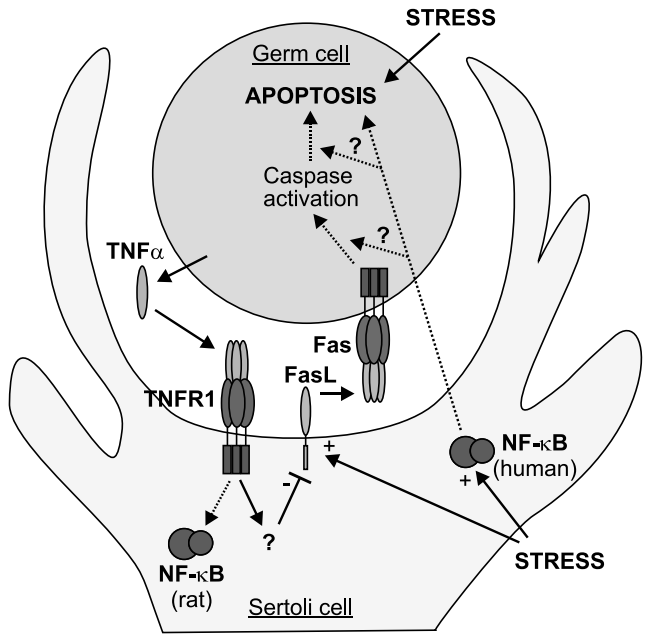

Fig. 10. Fas- and TNFR1-mediated signaling in the regulation of male germ cell death. The cartoon is based on results from several species, and species specific-differences in the pathways illustrated may exist. In several species, activation of germ cell Fas by FasL induces apoptosis of these cells. This apoptosis appears to be mediated by caspases, since in the human testis *in vitro* the broad-range caspase inhibitor Z-VAD.FMK prevents germ cell death. FasL appears to be constitutively expressed by the Sertoli cells. The testicular level of FasL is elevated during Sertoli cell injury and under exposure to external stress. In contrast to the death-inducing Fas system, the TNFα-mediated signaling appears to prevent germ cell death under apoptosis-inducing conditions. Germ cells produce TNFα, which most likely acts on TNFR1 on Sertoli cells. In cultured human seminiferous tubules, TNFα inhibits germ cell apoptosis and this effect is associated with down-regulation of the FasL. In the rat, but not in man, TNFα induces increased Sertoli cell NF-κB activity. In contrast, in the human testis, *in vitro* exposure to apoptosis-inducing conditions results in Sertoli cell activation of NF-κB, which may exert pro-apoptotic effects on germ cells.

Prevention of stress-induced male germ cell apoptosis by anti-inflammatory drugs

In the present study, several common anti-inflammatory drugs were found to suppress *in vitro*-induced death of male germ cells. The anti-apoptotic effect of sulfasalazine may, at least partly, be mediated through inhibition of Sertoli cell NF-κB. Because of the general anti-inflammatory action of SS, other simultaneous NF-κB-independent pathways directly affecting either the Sertoli cells or the germ cells may also contribute to sulfasalazine-mediated germ cell survival. Of the other anti-inflammatory drugs tested, aspirin and sulindac have previously been reported to inhibit NF-κB in some types of cell, whereas indomethasin should not affect NF-κB (186,187). All these compounds were, to some extent, able to inhibit germ cell death, but not NF-κB, in cultured seminiferous tubules. Thus, the anti-apoptotic effects of these compounds seem to be mediated by mechanisms other than Sertoli cell NF-κB inhibition and may be based on their other effects, such as cyclo-oxygenase inhibition, or on their antioxidative properties. Although this result could be interpreted as showing that NF-κB activation does not play an obligatory role in the induction of testicular apoptosis, it seems more likely to reflect the presence of many compensatory or parallel apoptotic pathways in the testis.

Irrespective of the mechanism of their anti-apoptotic effect, the anti-inflammatory drugs were able to inhibit stress-induced male germ cell death. Importantly, at least after the sulfasalazine and aspirin treatments, the overall tissue morphology appeared to be undisturbed. Moreover, the apoptotic death of meiotic and postmeiotic germ cells could be modulated by these drugs, with no hazardous effects on the Sertoli cells, which are needed for continuing spermatogenesis. Our results are supported by reports of reversible infertility and of sperm abnormalities, including abnormally large numbers of immature germ cells in the sperm, in men treated with sulfasalazine for inflammatory bowel diseases (229-233). These findings suggest that sulfasalazine also affects germ cell maturation and apoptosis *in vivo*. Moreover, the reversibility of the infertility supports our observation that only the apoptotic death of the germ cells in later phases of maturation is affected by the sulfasalazine treatment, leaving the Sertoli cells and the immature germ cells undisturbed and potentially capable of functional spermatogenesis after cessation of the treatment. In conclusion, our results obtained with anti-inflammatory drugs raise the possibility that they could be used therapeutically to prevent male germ cell death induced by transient testicular stress situations.

Conclusions and Future Prospects

The present *in vitro* studies on human testicular apoptosis suggest an important role for estrogens in the survival of male germ cells. Furthermore, they show that the signaling pathways initiated by the cellular death receptors Fas and TNFR1 are involved in the regulation of *in vitro*-induced male germ cell apoptosis. While the Fas system mediates germ cell death, the TNFα-induced signaling is associated with down-regulation of the Fas system and inhibition of germ cell apoptosis. Finally, the transcription factor NF-κB is suggested to regulate testicular apoptotic pathways which function in parallel with or downstream of that triggered by Fas. That the pathways described in the present studies regulate germ cell death in cultured human seminiferous tubules suggests that they also regulate germ cell apoptosis during spermatogenesis and are involved in increased germ cell death during testicular stress. Importantly, germ cell death was prevented by pharmacological modulation of the apoptotic pathways described in the present studies. Many of the compounds with anti-apoptotic properties are common anti-inflammatory drugs. These findings raise the possibility that male germ cell death could be prevented *in vivo* during situations involving excessive germ cell loss, such as during cancer therapies.

The potential use of anti-apoptotic compounds in humans *in vivo* requires careful evaluation of the capability of the seminiferous epithelium for functional spermatogenesis after the treatments. This evaluation should be done both in animal models and, in view of species specificity, also in a culture model of human seminiferous epithelium. Thus, in addition to studying the apoptosis-inhibiting capability of the candidate compounds, detailed morphological analysis of the cells of the seminiferous epithelium should be performed and continuous spermatogenesis should be assured. Since various disrupters, e.g. radiation or chemotherapy, may cause selective injury of either germ cells or supporting Sertoli cells, the ability of the individual compounds to prevent injury caused by such disrupters should be investigated. Finally, pharmacological inhibition of pathways that eliminate injured cells could lead to preservation of transformed cells, which could potentially cause tumorgenesis and transmission of mutations in the germ line. Accordingly, generation of healthy offspring after apoptosis-inhibiting treatments should be confirmed in studies employing experimental animals. Only after completion of these experiments should studies be initiated in humans *in vivo*.

Acknowledgments

This study was carried out at the Research Laboratory of the Hospital for Children and Adolescents at the University of Helsinki between 1997 and 2002. I am grateful to all those who have contributed to this work and to my friends and family for making this a great period of time. I would especially like to thank the following people:

I express my sincerest gratitude to Professor Jaakko Perheentupa and Professor Mikael Knip, the former and current Heads of the Hospital for Children and Adolescents, and to Docent Erkki Savilahti, the Head of the Research Laboratory of the Hospital for Children and Adolescents, for providing excellent research facilities.

I am most grateful to my supervisor Docent Leo Dunkel, who not only gave me an interesting topic and encouraged and guided me throughout the work, but also offered me an opportunity to pursue my own ideas and to work independently as a young scientist. I admire his wide knowledge on both clinical practice and basic science, his capacity of precise, fast and logical thinking and his enthusiasm when he becomes really interested in something. I also appreciate his patience when I was too much concerned over my plans and results. Thank you Leo for guiding me into the world of science!

I express my gratitude to Professor Martti Parvinen, who gave invaluable help with interpretation of the results concerning ISEL, immunohistochemistry and electron microscopy. His fascinating personality and enthusiasm were most inspiring to me and his expertise is gratefully appreciated.

Professor Ismo Virtanen and Professor John Eriksson are acknowledged for critically reviewing the manuscript and for their constructive comments. Docent Jorma Toppari and Docent Jorma Palvimo, the tutors appointed by the Helsinki Biomedical Graduate School, are warmly thanked for motivating and valuable comments during the work. I also sincerely acknowledge Professor Petri Kovanen, the Head of the Wihuri Research Institute, for interest in my work and for letting me to use their facilities whenever needed.

I owe my warmest gratitude to the people in our “apoptosis-group”. I am deeply indebted to Krista Erkkilä for setting up the culture model and for teaching me the basics of laboratory work, and I warmly thank her for sharing all the best moments of success and the disappointments of failures. Her positive attitude and commitment to this work were of great importance during the years. I thank Marjut Otala and Laura Suomalainen for pleasant co-operation and friendship, and especially for support during the hectic times before the birth of my firstborn son last spring. Virpi Ahokas and Kaisa Alasalmi are warmly acknowledged for their skillful and effective technical assistance

that has been invaluable to the work. I wish to also thank Merja Haukka and Riikka Rantakari, whose high quality technical assistance was of great help in the beginning of the work.

I wish to thank everybody in the Research Laboratory of the Hospital for Children and Adolescents for making the days in the lab enjoyable and productive. Professor Markku Heikinheimo is warmely thanked for his encouraging and positive attitude toward young scientists and for many inspiring conversations. My sincere thanks go to my colleagues Eeva, Tiina, Annuski, Mika, Minna, Mia, Ilkka, Sanne, Nina, Petra, Henrikka and Jaska for friendship, help and encouragement. I wish to address special thanks to Ilkka Ketola for his invaluable help with bringing and preparing the orchidectomy samples, to Eeva Martelin for guiding me with EMSAs, and to Mika Saksela for teaching me a number of new methods. I am grateful to all the personnel of the lab for always being ready to help despite their own duties. I extend my gratitude to Sinikka Heikkilä, who provided excellent samples for immunohistochemistry. The Stuff of the Department of Urology, Helsinki University Central Hospital, is also acknowledged for pleasant and effective co-operation.

Mrs. Jean Margaret Perttunen is cordially thanked for revision of the English language of the thesis and the original publications.

I am happy to have friends that have enriched my life and continuously encouraged me. Thank you all for your patience and sympathy!

I owe my deepest gratitude to my parents Eira and Jussi and to my sister Virpi for encouragement, support, and love and for always believing in me. I thank Harri for friendship and for interest in my work. My parents-in-law Leena and Pertti are warmly acknowledged for continuous interest in my work, for helpful advice, and for kind care. I also wish to thank all of you for giving me productive periods of work when you were taking care of Juho.

Finally, my deepest and loving thanks belong to my husband Markku and to our little son Juho for their love, support, and patience. I am privileged to have the most wonderful husband, who not only brings joy, happiness, and safety into my life, but also has generously shared his expertise in basic science and helped me with numerous problems. It was Markku's enthusiasm in science that aroused my interest in research and made this work possible.

The Finnish Medical Society Duodecim, the Foundation for Pediatric Research, the Helsinki Biomedical Graduate School, the EVO funds of Helsinki University Central Hospital, the Research and Science Foundation of Farmos, and the Sigrid Juselius Foundation are acknowledged for financial support.

Helsinki, June 2002

Virve Pentikäinen

References

1. de Kretser DM, Loveland KL, Meinhardt A, Simorangkir D, and Wreford N: Spermatogenesis. *Hum Reprod* 13 Suppl 1: 1-8, 1998.
2. Meachem S, von S, V, and Schlatt S: Spermatogonia: stem cells with a great perspective. *Reproduction* 121: 825-834, 2001.
3. Print CG and Loveland KL: Germ cell suicide: new insights into apoptosis during spermatogenesis. *Bioessays* 22: 423-430, 2000.
4. Sutton KA: Molecular mechanisms involved in the differentiation of spermatogenic stem cells. *Rev Reprod* 5: 93-98, 2000.
5. de Rooij DG: Proliferation and differentiation of spermatogonial stem cells. *Reproduction* 121: 347-354, 2001.
6. Dunkel L, Hirvonen V, and Erkkilä K: Clinical aspects of male germ cell apoptosis during testis development and spermatogenesis. *Cell Death Differ* 4 : 171-179, 1997.
7. Chemes HE: Infancy is not a quiescent period of testicular development. *Int J Androl* 24: 2-7, 2001.
8. Griswold MD: The central role of Sertoli cells in spermatogenesis. *Semin Cell Dev Biol* 9: 411-416, 1998.
9. Grootegoed JA, Siep M, and Baarends WM: Molecular and cellular mechanisms in spermatogenesis. *Baillieres Best Pract Res Clin Endocrinol Metab* 14: 331-343, 2000.
10. Clermont Y: The cycle of the seminiferous epithelium in man. *Am J Anat* 112: 35-51, 1963.
11. Parvinen M: Regulation of the seminiferous epithelium. *Endocr Rev* 3: 404-417, 1982.
12. Kerr JB: Functional cytology of the human testis. *Baillieres Clin Endocrinol Metab* 6: 235-250, 1992.
13. Spradling A, Drummond-Barbosa D, and Kai T: Stem cells find their niche. *Nature* 414: 98-104, 2001.
14. Arslan M, Weinbauer GF, Schlatt S, Shahab M, and Nieschlag E: FSH and testosterone, alone or in combination, initiate testicular growth and increase the number of spermatogonia and Sertoli cells in a juvenile non-human primate (Macaca mulatta). *J Endocrinol* 136: 235-243, 1993.
15. Clermont Y: Renewal of spermatogonia in man. *Am J Anat* 118: 509-524, 1966.
16. Hecht NB: Molecular mechanisms of male germ cell differentiation. *Bioessays* 20: 555-561, 1998.
17. Sassone-Corsi P: Transcriptional checkpoints determining the fate of male germ cells. *Cell* 88: 163-166, 1997.
18. Verhoeven G: Local control systems within the testis. *Baillieres Clin Endocrinol Metab* 6: 313-333, 1992.
19. Johnson KJ and Boekelheide K: Dynamic Testicular Adhesion Junctions Are Immunologically Unique. I. Localization of p120 Catenin in Rat Testis. *Biol Reprod* 66: 983-991, 2002.
20. Johnson KJ and Boekelheide K: Dynamic Testicular Adhesion Junctions Are Immunologically Unique. II. Localization of Classic Cadherins in Rat Testis. *Biol Reprod* 66: 992-1000, 2002.
21. Mulholland DJ, Dedhar S, and Vogl AW: Rat seminiferous epithelium contains a unique junction (Ectoplasmic specialization) with signaling properties both of cell/cell and cell/matrix junctions. *Biol Reprod* 64: 396-407, 2001.
22. Roscoe WA, Barr KJ, Mhawi AA, Pomerantz DK, and Kidder GM: Failure of spermatogenesis in mice lacking connexin43. *Biol Reprod* 65: 829-838, 2001.
23. Goldberg GS, Lampe PD, and Nicholson BJ: Selective transfer of endogenous metabolites through gap junctions composed of different connexins. *Nat Cell Biol* 1: 457-459, 1999.
24. Kam Y, Kim DY, Koo SK, and Joe CO: Transfer of second messengers through gap junction connexin 43 channels reconstituted in liposomes. *Biochim Biophys Acta* 1372: 384-388, 1998.

25. Krutovskikh VA, Piccoli C, and Yamasaki H: Gap junction intercellular communication propagates cell death in cancerous cells. *Oncogene* 21: 1989-1999, 2002.
26. Raff M: Cell suicide for beginners. *Nature* 396: 119-122, 1998.
27. Kaufmann SH and Hengartner MO: Programmed cell death: alive and well in the new millennium. *Trends Cell Biol* 11: 526-534, 2001.
28. Nagata S: Apoptotic DNA fragmentation. *Exp Cell Res* 256: 12-18, 2000.
29. Nicholson DW and Thornberry NA: Caspases: killer proteases. *Trends Biochem Sci* 22: 299-306, 1997.
30. Hengartner MO: The biochemistry of apoptosis. *Nature* 407: 770-776, 2000.
31. Gupta S: Molecular steps of death receptor and mitochondrial pathways of apoptosis. *Life Sci* 69: 2957-2964, 2001.
32. Walczak H and Krammer PH: The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. *Exp Cell Res* 256: 58-66, 2000.
33. Kroemer G and Reed JC: Mitochondrial control of cell death. *Nat Med* 6: 513-519, 2000.
34. Adrain C and Martin SJ: The mitochondrial apoptosome: a killer unleashed by the cytochrome seas. *Trends Biochem Sci* 26: 390-397, 2001.
35. Antonsson B and Martinou JC: The Bcl-2 protein family. *Exp Cell Res* 256: 50-57, 2000.
36. Kirsch DG, Doseff A, Chau BN, Lim DS, Souza-Pinto NC, Hansford R, Kastan MB, Lazebnik YA, and Hardwick JM: Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c. *J Biol Chem* 274: 21155-21161, 1999.
37. Levine AJ: p53, the cellular gatekeeper for growth and division. *Cell* 88: 323-331, 1997.
38. Moll UM and Zaika A: Nuclear and mitochondrial apoptotic pathways of p53. *FEBS Lett* 493: 65-69, 2001.
39. Vousden KH: p53: Death star. *Cell* 103: 691-694, 2000.
40. Barkett M and Gilmore TD: Control of apoptosis by Rel/NF-κB transcription factors. *Oncogene* 18: 6910-6924, 1999.
41. Karin M, Liu Z, and Zandi E: AP-1 function and regulation. *Curr Opin Cell Biol* 9: 240-246, 1997.
42. Leppä S and Bohmann D: Diverse functions of JNK signaling and c-Jun in stress response and apoptosis. *Oncogene* 18: 6158-6162, 1999.
43. Pahl HL: Activators and target genes of Rel/NF-κB transcription factors. *Oncogene* 18: 6853-6866, 1999.
44. Ashkenazi A and Dixit VM: Death receptors: signaling and modulation. *Science* 281: 1305-1308, 1998.
45. Baud V and Karin M: Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol* 11: 372-377, 2001.
46. Garrido C, Gurbuxani S, Ravagnan L, and Kroemer G: Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochem Biophys Res Commun* 286: 433-442, 2001.
47. Xanthoudakis S and Nicholson DW: Heat-shock proteins as death determinants. *Nat Cell Biol* 2: E163-E165, 2000.
48. Ohanian J and Ohanian V: Sphingolipids in mammalian cell signalling. *Cell Mol Life Sci* 58: 2053-2068, 2001.
49. Hannun YA and Luberto C: Ceramide in the eukaryotic stress response. *Trends Cell Biol* 10: 73-80, 2000.
50. Pyne S and Pyne NJ: Sphingosine 1-phosphate signalling in mammalian cells. *Biochem J* 349: 385-402, 2000.
51. Xiang J, Chao DT, and Korsmeyer SJ: BAX-induced cell death may not require interleukin 1 beta-converting enzyme-like proteases. *Proc Natl Acad Sci U S A* 93: 14559-14563, 1996.

52. Nylandsted J, Rohde M, Brand K, Bastholm L, Elling F, and Jäättelä M: Selective depletion of heat shock protein 70 (Hsp70) activates a tumor- specific death program that is independent of caspases and bypasses Bcl- 2. *Proc Natl Acad Sci U S A* 97: 7871-7876, 2000.
53. Rodriguez I, Ody C, Araki K, Garcia I, and Vassalli P: An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. *EMBO J* 16: 2262-2270, 1997.
54. Yan W, Suominen J, Samson M, Jegou B, and Toppari J: Involvement of Bcl-2 family proteins in germ cell apoptosis during testicular development in the rat and pro-survival effect of stem cell factor on germ cells in vitro. *Mol Cell Endocrinol* 165: 115-129, 2000.
55. Bartke A: Apoptosis of male germ cells, a generalized or a cell type-specific phenomenon? *Endocrinology* 136: 3-4, 1995.
56. Clermont Y: Quantitative analysis of spermatogenesis of the rat: a revised model for the renewal of spermatogonia. *Am J Anat* 111: 111-129, 1962.
57. Huckins C: The morphology and kinetics of spermatogonial degeneration in normal adult rats: an analysis using a simplified classification of the germinal epithelium. *Anat Rec* 190: 905-926, 1978.
58. Sinha Hikim AP and Swerdloff RS: Hormonal and genetic control of germ cell apoptosis in the testis. *Rev Reprod* 4: 38-47, 1999.
59. Braun RE: Every sperm is sacred—or is it? *Nat Genet* 18: 202-204, 1998.
60. Odorisio T, Rodriguez TA, Evans EP, Clarke AR, and Burgoyne PS: The meiotic checkpoint monitoring synapsis eliminates spermatocytes via p53-independent apoptosis. *Nat Genet* 18: 257-261, 1998.
61. Dunkel L, Taskinen S, Hovatta O, Tilly JL, and Wikstrom S: Germ cell apoptosis after treatment of cryptorchidism with human chorionic gonadotropin is associated with impaired reproductive function in the adult. *J Clin Invest* 100: 2341-2346, 1997.
62. Henriksen K, Hakovirta H, and Parvinen M: In-situ quantification of stage-specific apoptosis in the rat seminiferous epithelium: effects of short-term experimental cryptorchidism. *Int J Androl* 18: 256-262, 1995.
63. Lysiak JJ, Turner SD, and Turner TT: Molecular pathway of germ cell apoptosis following ischemia/ reperfusion of the rat testis. *Biol Reprod* 63: 1465-1472, 2000.
64. Shikone T, Billig H, and Hsueh AJ: Experimentally induced cryptorchidism increases apoptosis in rat testis. *Biol Reprod* 51: 865-872, 1994.
65. Tesarik J, Greco E, Cohen-Bacrie P, and Mendoza C: Germ cell apoptosis in men with complete and incomplete spermiogenesis failure. *Mol Hum Reprod* 4: 757-762, 1998.
66. Blanchard KT, Allard EK, and Boekelheide K: Fate of germ cells in 2,5-hexanedione-induced testicular injury. I. Apoptosis is the mechanism of germ cell death. *Toxicol Appl Pharmacol* 137: 141-148, 1996.
67. Henriksen K, Kulmala J, Toppari J, Mehrotra K, and Parvinen M: Stage-specific apoptosis in the rat seminiferous epithelium: quantification of irradiation effects. *J Androl* 17: 394-402, 1996.
68. Hikim AP, Wang C, Leung A, and Swerdloff RS: Involvement of apoptosis in the induction of germ cell degeneration in adult rats after gonadotropin-releasing hormone antagonist treatment. *Endocrinology* 136: 2770-2775, 1995.
69. Richburg JH and Boekelheide K: Mono-(2-ethylhexyl) phthalate rapidly alters both Sertoli cell vimentin filaments and germ cell apoptosis in young rat testes. *Toxicol Appl Pharmacol* 137: 42-50, 1996.
70. Tapanainen JS, Tilly JL, Vihko KK, and Hsueh AJ: Hormonal control of apoptotic cell death in the testis: gonadotropins and androgens as testicular cell survival factors. *Mol Endocrinol* 7: 643-650, 1993.
71. Erkkilä K, Henriksen K, Hirvonen V, Rannikko S, Salo J, Parvinen M, and Dunkel L: Testosterone regulates apoptosis in adult human seminiferous tubules *in vitro*. *J Clin Endocrinol Metab* 82: 2314-2321, 1997.

72. Henriksen K, Kangasniemi M, Parvinen M, Kaipia A, and Hakovirta H: In vitro, follicle-stimulating hormone prevents apoptosis and stimulates deoxyribonucleic acid synthesis in the rat seminiferous epithelium in a stage-specific fashion. *Endocrinology* 137: 2141-2149, 1996.
73. Knudson CM, Tung KS, Tourtellotte WG, Brown GA, and Korsmeyer SJ: Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* 270: 96-99, 1995.
74. Yan W, Samson M, Jegou B, and Toppari J: Bcl-w forms complexes with Bax and Bak, and elevated ratios of Bax/Bcl-w and Bak/Bcl-w correspond to spermatogonial and spermatocyte apoptosis in the testis. *Mol Endocrinol* 14: 682-699, 2000.
75. Print CG, Loveland KL, Gibson L, Meehan T, Stylianou A, Wreford N, de Kretser D, Metcalf D, Kontgen F, Adams JM, and Cory S: Apoptosis regulator bcl-w is essential for spermatogenesis but appears otherwise redundant. *Proc Natl Acad Sci U S A* 95: 12424-12431, 1998.
76. Ross AJ, Waymire KG, Moss JE, Parlow AF, Skinner MK, Russell LD, and MacGregor GR: Testicular degeneration in Bclw-deficient mice. *Nat Genet* 18: 251-256, 1998.
77. Russell LD, Warren J, Debeljuk L, Richardson LL, Mahar PL, Waymire KG, Amy SP, Ross AJ, and MacGregor GR: Spermatogenesis in Bclw-deficient mice. *Biol Reprod* 65: 318-332, 2001.
78. Beumer TL, Roepers-Gajadien HL, Gademan IS, van Buul PP, Gil-Gomez G, Rutgers DH, and de Rooij DG: The role of the tumor suppressor p53 in spermatogenesis. *Cell Death Differ* 5: 669-677, 1998.
79. Hasegawa M, Zhang Y, Niibe H, Terry NH, and Meistrich ML: Resistance of differentiating spermatogonia to radiation-induced apoptosis and loss in p53-deficient mice. *Radiat Res* 149: 263-270, 1998.
80. Yin Y, DeWolf WC, and Morgentaler A: Experimental cryptorchidism induces testicular germ cell apoptosis by p53-dependent and -independent pathways in mice. *Biol Reprod* 58: 492-496, 1998.
81. Allemand I, Anglo A, Jeantet AY, Cerutti I, and May E: Testicular wild-type p53 expression in transgenic mice induces spermiogenesis alterations ranging from differentiation defects to apoptosis. *Oncogene* 18: 6521-6530, 1999.
82. Eddy EM: Role of heat shock protein HSP70-2 in spermatogenesis. *Rev Reprod* 4: 23-30, 1999.
83. Billig H, Furuta I, Rivier C, Tapanainen J, Parvinen M, and Hsueh AJ: Apoptosis in testis germ cells: developmental changes in gonadotropin dependence and localization to selective tubule stages. *Endocrinology* 136: 5-12, 1995.
84. Shetty J, Marathe GK, and Dighe RR: Specific immunoneutralization of FSH leads to apoptotic cell death of the pachytene spermatocytes and spermatogonial cells in the rat. *Endocrinology* 137: 2179-2182, 1996.
85. Brinkworth MH, Weinbauer GF, Schlatt S, and Nieschlag E: Identification of male germ cells undergoing apoptosis in adult rats. *J Reprod Fertil* 105: 25-33, 1995.
86. Henriksen K, Hakovirta H, and Parvinen M: Testosterone inhibits and induces apoptosis in rat seminiferous tubules in a stage-specific manner: in situ quantification in squash preparations after administration of ethane dimethane sulfonate. *Endocrinology* 136: 3285-3291, 1995.
87. Woolveridge I, Boer-Brouwer M, Taylor MF, Teerds KJ, Wu FC, and Morris ID: Apoptosis in the rat spermatogenic epithelium following androgen withdrawal: changes in apoptosis-related genes. *Biol Reprod* 60: 461-470, 1999.
88. McLachlan RI: The endocrine control of spermatogenesis. *Baillieres Best Pract Res Clin Endocrinol Metab* 14: 345-362, 2000.
89. Suarez-Quian CA, Martinez-Garcia F, Nistal M, and Regadera J: Androgen receptor distribution in adult human testis. *J Clin Endocrinol Metab* 84: 350-358, 1999.
90. Yan W, Suominen J, and Toppari J: Stem cell factor protects germ cells from apoptosis in vitro. *J Cell Sci* 113 (Pt 1): 161-168, 2000.

91. Jones ME and Simpson ER: Oestrogens in male reproduction. *Baillieres Best Pract Res Clin Endocrinol Metab* 14: 505-516, 2000.
92. O'Donnell L, Robertson KM, Jones ME, and Simpson ER: Estrogen and spermatogenesis. *Endocr Rev* 22: 289-318, 2001.
93. Almadhidi J, Séralini GE, Fresnel J, Silberzahn P, and Gaillard JL: Immunohistochemical localization of cytochrome P450 aromatase in equine gonads. *J Histochem Cytochem* 43: 571-577, 1995.
94. Kwon S, Hess RA, Bunick D, Nitta H, Janulis L, Osawa Y, and Bahr JM: Rooster testicular germ cells and epididymal sperm contain P450 aromatase. *Biol Reprod* 53: 1259-1264, 1995.
95. Tsubota T, Nitta H, Osawa Y, Mason IJ, Kita I, Tiba T, and Bahr JM: Immunolocalization of steroidogenic enzymes $P450_{scc}$ 3b-HSD $P450_{c17}$ and $P450_{arom}$ in the hokkaido brown bear (*Ursus arctos yesoensis*) testis. *Gen Comp Endocrinol* 92: 439-444, 1993.
96. Luconi M, Bonaccorsi L, Forti G, and Baldi E: Effects of estrogenic compounds on human spermatozoa: evidence for interaction with a nongenomic receptor for estrogen on human sperm membrane. *Mol Cell Endocrinol* 178: 39-45, 2001.
97. Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB, and Korach KS: Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology* 137: 4796-4805, 1996.
98. Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS, and Lubahn DB: A role for oestrogens in the male reproductive system. *Nature* 390: 509-512, 1997.
99. Robertson KM, O'Donnell L, Jones ME, Meachem SJ, Boon WC, Fisher CR, Graves KH, McLachlan RI, and Simpson ER: Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. *Proc Natl Acad Sci U S A* 96: 7986-7991, 1999.
100. Krege JH, Hodgin JB, Couse JF, Enmark E , Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, and Smithies O: Generation and reproductive phenotypes of mice lacking estrogen receptor β. *Proc Natl Acad Sci U S A* 95: 15677-15682, 1998.
101. Couse JF, Hewitt SC, Bunch DO, Sar M, Walker VR, Davis BJ, and Korach KS: Postnatal sex reversal of the ovaries in mice lacking estrogen receptors α and β. *Science* 286: 2328-2331, 1999.
102. Smith EP, Boyd J, Frank GR, Takahashi H , Cohen RM, Specker B, Williams TC, Lubahn DB, and Korach KS: Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *N Engl J Med* 331: 1056-1061, 1994.
103. Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, and Simpson ER: Effect of testosterone and estradiol in a man with aromatase deficiency. *N Engl J Med* 337 : 91-95, 1997.
104. Nagata S and Golstein P: The Fas death factor. *Science* 267: 1449-1456, 1995.
105. Schneider P and Tschopp J: Apoptosis induced by death receptors . *Pharm Acta Helv* 74: 281-286, 2000.
106. Itoh N, Yonehara S, Ishii A, Yonehara M , Mizushima S, Sameshima M, Hase A, Seto Y, and Nagata S: The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* 66: 233-243, 1991.
107. Oehm A, Behrmann I, Falk W, Pawlita M, Maier G, Klas C, Li-Weber M, Richards S, Dhein J, Trauth BC, and .: Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. Sequence identity with the Fas antigen. *J Biol Chem* 267: 10709-10715, 1992.
108. Cascino I, Fiucci G, Papoff G, and Ruberti G: Three functional soluble forms of the human apoptosis-inducing Fas molecule are produced by alternative splicing. *J Immunol* 154: 2706-2713, 1995.
109. Cheng J, Zhou T, Liu C, Shapiro JP, Brauer MJ, Kiefer MC, Barr PJ, and Mountz JD: Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule . *Science* 263: 1759-1762, 1994.

110. Suda T, Takahashi T, Golstein P, and Nagata S: Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* 75: 1169-1178, 1993.
111. Suda T, Hashimoto H, Tanaka M, Ochi T, and Nagata S: Membrane Fas ligand kills human peripheral blood T lymphocytes, and soluble Fas ligand blocks the killing. *J Exp Med* 186: 2045-2050, 1997.
112. Tanaka M, Itai T, Adachi M, and Nagata S: Downregulation of Fas ligand by shedding. *Nat Med* 4: 31-36, 1998.
113. Tanaka M, Suda T, Haze K, Nakamura N, Sato K, Kimura F, Motoyoshi K, Mizuki M, Tagawa S, Ohga S, Hatake K, Drummond AH, and Nagata S: Fas ligand in human serum. *Nat Med* 2: 317-322, 1996.
114. Nagata S: Apoptosis by death factor. *Cell* 88: 355-365, 1997.
115. Bellgrau D, Gold D, Selawry H, Moore J, Franzusoff A, and Duke RC: A role for CD95 ligand in preventing graft rejection. *Nature* 377: 630-632, 1995.
116. Aggarwal BB, Samanta A, and Feldman M: TNF receptors. In *Cytokine reference*. Oppenheim JJ and Feldman M eds. Academic press, San Diego. 2000, 1619-1632.
117. Fiers W: Tumor necrosis factor. Characterization at the molecular, cellular and *in vivo* level. *FEBS Lett* 285: 199-212, 1991.
118. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, and Cerretti DP: A metalloproteinase disintegrin that releases tumour-necrosis factor- α from cells. *Nature* 385: 729-733, 1997.
119. Moss ML, Jin SL, Milla ME, Bickett DM, Burkhart W, Carter HL, Chen WJ, Clay WC, Didsbury JR, Hassler D, Hoffman CR, Kost TA, Lambert MH, Leesnitzer MA, McCauley P, McGeehan G, Mitchell J, Moyer M, Pahel G, Rocque W, Overton LK, Schoenen F, Seaton T, Su JL, and Becherer JD: Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-α. *Nature* 385: 733-736, 1997.
120. Baker SJ and Reddy EP: Modulation of life and death by the TNF receptor superfamily. *Oncogene* 17: 3261-3270, 1998.
121. Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, and Lenardo MJ: A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science* 288: 2351-2354, 2000.
122. Siegel RM, Frederiksen JK, Zacharias DA, Chan FK, Johnson M, Lynch D, Tsien RY, and Lenardo MJ: Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. *Science* 288: 2354-2357, 2000.
123. Krammer PH: CD95's deadly mission in the immune system. *Nature* 407: 789-795, 2000.
124. Nagata S: Fas ligand-induced apoptosis. *Annu Rev Genet* 33: 29-55, 1999.
125. Davis RJ: Signal transduction by the JNK group of MAP kinases. *Cell* 103: 239-252, 2000.
126. Holmstrom TH and Eriksson JE: Phosphorylation-Based signaling in Fas receptor-mediated apoptosis. *Crit Rev Immunol* 20: 121-152, 2000.
127. He T, Stepulak A, Holmstrom TH, Omary MB, and Eriksson JE: The intermediate filament protein keratin 8 is a novel cytoplasmic substrate for c-Jun N-terminal kinase. *J Biol Chem* 277: 10767-10774, 2002.
128. Holmstrom TH, Schmitz I, Soderstrom TS, Poukkula M, Johnson VL, Chow SC , Krammer PH, and Eriksson JE: MAPK/ERK signaling in activated T cells inhibits CD95/Fas-mediated apoptosis downstream of DISC assembly. *EMBO J* 19: 5418-5428, 2000.
129. Tran SE, Holmstrom TH, Ahonen M, Kahari VM, and Eriksson JE: MAPK/ERK overrides the apoptotic signaling from Fas, TNF, and TRAIL receptors. *J Biol Chem* 276: 16484-16490, 2001.
130. Siegel RM, Chan FK, Chun HJ, and Lenardo MJ: The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nat Immunol* 1: 469-474, 2000.

131. Restifo NP: Not so Fas: Re-evaluating the mechanisms of immune privilege and tumor escape. *Nat Med* 6: 493-495, 2000.
132. Beutler B: TNF, immunity and inflammatory disease: lessons of the past decade. *J Investig Med* 43: 227-235, 1995.
133. Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, and Boldin MP: Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu Rev Immunol* 17: 331-367, 1999.
134. Baldwin AS, Jr.: The NF-κB and IκB proteins: new discoveries and insights. *Annu Rev Immunol* 14: 649-683, 1996.
135. Ledgerwood EC, Pober JS, and Bradley JR: Recent advances in the molecular basis of TNF signal transduction. *Lab Invest* 79: 1041-1050, 1999.
136. Sugarman BJ, Aggarwal BB, Hass PE, Figari IS, Palladino MA, Jr., and Shepard HM: Recombinant human tumor necrosis factor-alpha: effects on proliferation of normal and transformed cells *in vitro*. *Science* 230: 943-945, 1985.
137. Wallach D: Cell death induction by TNF: a matter of self control. *Trends Biochem Sci* 22: 107-109, 1997.
138. Sugihara A, Saiki S, Tsuji M, Tsujimura T, Nakata Y, Kubota A, Kotake T, and Terada N: Expression of Fas and Fas ligand in the testes and testicular germ cell tumors: an immunohistochemical study. *Anticancer Res* 17: 3861-3865, 1997.
139. Xerri L, Devilard E, Hassoun J, Mawas C , and Birg F: Fas ligand is not only expressed in immune privileged human organs but is also coexpressed with Fas in various epithelial tissues. *Mol Pathol* 50: 87-91, 1997.
140. Lee J, Richburg JH, Younkin SC, and Boekelheide K: The Fas system is a key regulator of germ cell apoptosis in the testis. *Endocrinology* 138: 2081-2088, 1997.
141. Riccioli A, Starace D, D'Alessio A, Starace G, Padula F, De Cesaris P, Filippini A, and Ziparo E : TNF-α and IFN-γ regulate expression and function of the Fas system in the seminiferous epithelium. *J Immunol* 165: 743-749, 2000.
142. Lee J, Richburg JH, Shipp EB, Meistrich ML, and Boekelheide K: The Fas system, a regulator of testicular germ cell apoptosis, is differentially up-regulated in Sertoli cell versus germ cell injury of the testis. *Endocrinology* 140: 852-858, 1999.
143. De SK, Chen HL, Pace JL, Hunt JS, Terranova PF, and Enders GC: Expression of tumor necrosis factor-α in mouse spermatogenic cells. *Endocrinology* 133: 389-396, 1993.
144. Moore C and Hutson JC: Physiological relevance of tumor necrosis factor in mediating macrophage-Leydig cell interactions. *Endocrinology* 134: 63-69, 1994.
145. Xiong Y and Hales DB: Expression, regulation, and production of tumor necrosis factor-α in mouse testicular interstitial macrophages in vitro. *Endocrinology* 133: 2568-2573, 1993.
146. De Cesaris P, Starace D, Starace G, Filippini A, Stefanini M, and Ziparo E: Activation of Jun N-terminal kinase/stress-activated protein kinase pathway by tumor necrosis factor α leads to intercellular adhesion molecule-1 expression. *J Biol Chem* 274: 28978-28982, 1999.
147. Mauduit C, Besset V, Caussanel V, and Benahmed M: Tumor necrosis factor α receptor p55 is under hormonal (follicle- stimulating hormone) control in testicular Sertoli cells. *Biochem Biophys Res Commun* 224: 631-637, 1996.
148. Mauduit C, Gasnier F, Rey C, Chauvin MA , Stocco DM, Louisot P, and Benahmed M: Tumor necrosis factor-α inhibits Leydig cell steroidogenesis through a decrease in steroidogenic acute regulatory protein expression. *Endocrinology* 139: 2863-2868, 1998.
149. De Cesaris P, Starace D, Riccioli A, Padula F, Filippini A, and Ziparo E: Tumor necrosis factor-α induces interleukin-6 production and integrin ligand expression by distinct transduction pathways. *J Biol Chem* 273: 7566-7571, 1998.

150. Stéphan JP, Syed V, and Jégou B: Regulation of Sertoli cell IL-1 and IL-6 production *in vitro*. *Mol Cell Endocrinol* 134: 109-118, 1997.
151. Boussouar F, Grataroli R, Ji J, and Benahmed M: Tumor necrosis factor-α stimulates lactate dehydrogenase A expression in porcine cultured Sertoli cells: mechanisms of action. *Endocrinology* 140: 3054-3062, 1999.
152. Nehar D, Mauduit C, Boussouar F, and Benahmed M: Tumor necrosis factor-α-stimulated lactate production is linked to lactate dehydrogenase A expression and activity increase in porcine cultured Sertoli cells. *Endocrinology* 138: 1964-1971, 1997.
153. Sigillo F, Guillou F, Fontaine I, Benahmed M, and Magueresse-Battistoni B: *In vitro* regulation of rat Sertoli cell transferrin expression by tumor necrosis factor alpha and retinoic acid. *Mol Cell Endocrinol* 148: 163-170, 1999.
154. Delfino FJ and Walker WH: NF-κB induces cAMP-response element-binding protein gene transcription in Sertoli cells. *J Biol Chem* 274: 35607-35613, 1999.
155. Besset V, Magueresse-Battistoni B, Collette J, and Benahmed M: Tumor necrosis factor α stimulates insulin-like growth factor binding protein 3 expression in cultured porcine Sertoli cells. *Endocrinology* 137: 296-303, 1996.
156. Budnik LT, Jahner D, and Mukhopadhyay AK: Inhibitory effects of TNFα on mouse tumor Leydig cells: possible role of ceramide in the mechanism of action. *Mol Cell Endocrinol* 150: 39-46, 1999.
157. Li X, Youngblood GL, Payne AH, and Hales DB: Tumor necrosis factor-α inhibition of 17 alpha-hydroxylase/C17-20 lyase gene (Cyp17) expression. *Endocrinology* 136: 3519-3526, 1995.
158. Gilmore TD: The Rel/NF-κB signal transduction pathway: introduction. *Oncogene* 18: 6842-6844, 1999.
159. Whiteside ST and Israel A: IκB proteins: structure, function and regulation. *Semin Cancer Biol* 8: 75-82, 1997.
160. Karin M: How NF-κB is activated: the role of the Iκ B kinase (IKK) complex. *Oncogene* 18: 6867-6874, 1999.
161. Arenzana-Seisdedos F, Turpin P, Rodriguez M, Thomas D, Hay RT, Virelizier JL, and Dargemont C: Nuclear localization of IκBα promotes active transport of NF- κB from the nucleus to the cytoplasm. *J Cell Sci* 110 (Pt 3): 369-378, 1997.
162. Sun SC, Ganchi PA, Ballard DW, and Greene WC: NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. *Science* 259: 1912-1915, 1993.
163. Beg AA and Baltimore D: An essential role for NF-κB in preventing TNFα-induced cell death. *Science* 274: 782-784, 1996.
164. Liu ZG, Hsu H, Goeddel DV, and Karin M: Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. *Cell* 87: 565-576, 1996.
165. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, and Verma IM: Suppression of TNFα-induced apoptosis by NF-κB. *Science* 274: 787-789, 1996.
166. Wang CY, Mayo MW, and Baldwin AS: TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. *Science* 274: 784-787, 1996.
167. Wang CY, Cusack JC, Liu R, and Baldwin AS: Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-κB. *Nat Med* 5: 412-417, 1999.
168. Grimm S, Bauer MK, Baeuerle PA, and Schulze-Osthoff K: Bcl-2 down-regulates the activity of transcription factor NF-κB induced upon apoptosis. *J Cell Biol* 134: 13-23, 1996.
169. Hettmann T, DiDonato J, Karin M, and Leiden JM: An essential role for nuclear factor κB in promoting double positive thymocyte apoptosis. *J Exp Med* 189: 145-158, 1999.

170. Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahboubi A, and Green DR: DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1. *Mol Cell* 1: 543-551, 1998.
171. Kasibhatla S, Genestier L, and Green DR: Regulation of fas-ligand expression during activation-induced cell death in T lymphocytes via nuclear factor κB. *J Biol Chem* 274: 987-992, 1999.
172. Lawrence T, Gilroy DW, Colville-Nash PR, and Willoughby DA: Possible new role for NF-κB in the resolution of inflammation. *Nat Med* 7: 1291-1297, 2001.
173. Kaltschmidt B, Kaltschmidt C, Hofmann TG, Hehner SP, Droge W, and Schmitz ML: The pro- or anti-apoptotic function of NFκB is determined by the nature of the apoptotic stimulus. *Eur J Biochem* 267: 3828-3835, 2000.
174. Delfino F and Walker WH: Stage-specific nuclear expression of NF-κB in mammalian testis. *Mol Endocrinol* 12: 1696-1707, 1998.
175. Delfino F and Walker WH: Hormonal regulation of the NF-κB signaling pathway. *Mol Cell Endocrinol* 157: 1-9, 1999.
176. Parvinen M and Hecht NB: Identification of living spermatogenic cells of the mouse by transillumination-phase contrast microscopic technique for *'in situ'* analyses of DNA polymerase activities. *Histochemistry* 71: 567-579, 1981.
177. Han Y and Brasier A: Mechanism for biphasic RelA NF-κB1 nuclear translocation in tumor necrosis factor α -stimulated hepatocytes. *J Biol Chem* 272: 9825-9832, 1997.
178. Baird DT, Galbraith A, Fraser IS, and Newsam JE: The concentration of oestrone and oestradiol-17 in spermatic venous blood in man. *J Endocrinol* 57: 285-288, 1973.
179. Longcope C, Widrich W, and Sawin CT: The secretion of estrone and estradiol-17 by human testis. *Steroids* 20: 439-448, 1972.
180. Tamm J, Volkwein U, Kurniawan E, and Becker H: Concentrations of unconjugated 5α -androstane-3α, 17β-diol and 5α-androstane-3β, 17β-diol and their precursor in human testicular tissue. Comparison with testosterone, 5α- dihydrotestosterone, estradiol-17β and with steroid concentrations in human epididymis. *J Steroid Biochem* 26: 345-348, 1987.
181. Weinstein RL, Kelch RP, Jenner MR, Kaplan SL, and Grumbach MM: Secretion of unconjugated androgens and estrogens by the normal and abnormal human testis before and after human chorionic gonadotropin. *J Clin Invest* 53: 1-6, 1974.
182. Kiener PA, Davis PM, Starling GC, Mehlin C, Klebanoff SJ, Ledbetter JA, and Liles WC: Differential induction of apoptosis by Fas-Fas ligand interactions in human monocytes and macrophages. *J Exp Med* 185: 1511-1516, 1997.
183. Wahl C, Liptay S, Adler G, and Schmid RM: Sulfasalazine: a potent and specific inhibitor of nuclear factor κB. *J Clin Invest* 101: 1163-1174, 1998.
184. Weber CK, Liptay S, Wirth T, Adler G, and Schmid RM: Suppression of NF-κB activity by sulfasalazine is mediated by direct inhibition of IκB kinases α and β. *Gastroenterology* 119: 1209-1218, 2000.
185. Matsui K, Fine A, Zhu B, Marshak-Rothstein A, and Ju ST: Identification of two NF-κ B sites in mouse CD95 ligand (Fas ligand) promoter: functional analysis in T cell hybridoma. *J Immunol* 161: 3469-3473, 1998.
186. Yamamoto Y, Yin MJ, Lin KM, and Gaynor RB: Sulindac inhibits activation of the NF-κB pathway. *J Biol Chem* 274: 27307-27314, 1999.
187. Yin MJ, Yamamoto Y, and Gaynor RB: The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-beta. *Nature* 396: 77-80, 1998.
188. Hasegawa M, Wilson G, Russell LD, and Meistrich ML: Radiation-induced cell death in the mouse testis: relationship to apoptosis. *Radiat Res* 147: 457-467, 1997.

189. Fujioka H, Fujisawa M, Tatsumi N, Kanzaki M, Okuda Y, Okada H, Arakawa S, and Kamidono S: Sertoli cells inhibited apoptosis of pachytene spermatocytes and round spermatids. *Endocr Res* 27: 75-90, 2001.
190. Couse JF, Mahato D, Eddy EM, and Korach KS: Molecular mechanism of estrogen action in the male: insights from the estrogen receptor null mice. *Reprod Fertil Dev* 13: 211-219, 2001.
191. Ronco AM, Pino AM, and Valladares LE: RNA synthesis in the Leydig cells of the mature rat: effect of oestradiol-17 beta . *J Endocrinol* 116: 387-392, 1988.
192. Sairam MR and Berman MI: Direct inhibitory effects of estrogens on rat Leydig cells in vitro. *Steroids* 33: 233-241, 1979.
193. Tsai-Morris CH, Knox G, Luna S, and Dufau ML: Acquisition of estradiol-mediated regulatory mechanism of steroidogenesis in cultured fetal rat Leydig cells. *J Biol Chem* 261: 3471-3474, 1986.
194. Yanaihara T, Troen P, Troen BR, and Troen ML: Studies of the human testis. 3. Effect of estrogen on testosterone formation in human testis in vitro. *J Clin Endocrinol Metab* 34: 968-973, 1972.
195. Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, and Scanlan TS: Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. *Science* 277: 1508-1510, 1997.
196. Kelly MJ and Levin ER: Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol Metab* 12: 152-156, 2001.
197. Levin ER: Cell localization, physiology, and nongenomic actions of estrogen receptors. *J Appl Physiol* 91: 1860-1867, 2001.
198. Nadal A, Ropero AB, Fuentes E, and Soria B: The plasma membrane estrogen receptor: nuclear or unclear? *Trends Pharmacol Sci* 22: 597-599, 2001.
199. Zheng J and Ramirez VD: Rapid inhibition of rat brain mitochondrial proton F0F1-ATPase activity by estrogens: comparison with Na+, K+ -ATPase of porcine cortex. *Eur J Pharmacol* 368: 95-102, 1999.
200. Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M, and Gustafsson JA: Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J Clin Endocrinol Metab* 82: 4258-4265, 1997.
201. Pelletier G, Labrie C, and Labrie F: Localization of oestrogen receptor α, oestrogen receptor β and androgen receptors in the rat reproductive organs. *J Endocrinol* 165: 359-370, 2000.
202. Pavao M and Traish AM: Estrogen receptor antibodies: specificity and utility in detection, localization and analyses of estrogen receptor α and β. *Steroids* 66: 1-16, 2001.
203. Mahato D, Goulding EH, Korach KS, and Eddy EM: Estrogen receptor-α is required by the supporting somatic cells for spermatogenesis. *Mol Cell Endocrinol* 178: 57-63, 2001.
204. Adamopoulos DA, Lawrence DM, Vassilopoulos P, Kontogeorgos L, Ntounis A, and Swyer GI: Testosterone concentration in different functional compartments of the male reproductive tract. *Arch Androl* 3: 13-18, 1979.
205. Fukutani K, Isurugi K, Ishida H, and Yokoyama M: Effects of estrogens on the testosterone levels of peripheral and spermatic vein blood in patients with prostatic cancer. *J Urol* 122: 624-627, 1979.
206. Morse HC, Horike N, Rowley MJ, and Heller CG: Testosterone concentrations in testes of normal men: effects of testosterone propionate administration. *J Clin Endocrinol Metab* 37: 882-886, 1973.
207. Janssen PJ, Brinkmann AO, Boersma WJ, and Van der Kwast TH: Immunohistochemical detection of the androgen receptor with monoclonal antibody F39.4 in routinely processed, paraffin-embedded human tissues after microwave pre-treatment. *J Histochem Cytochem* 42: 1169-1175, 1994.

208. Kimura N, Mizokami A, Oonuma T, Sasano H, and Nagura H: Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues. *J Histochem Cytochem* 41: 671-678, 1993.
209. Vornberger W, Prins G, Musto NA, and Suarez-Quian CA: Androgen receptor distribution in rat testis: new implications for androgen regulation of spermatogenesis. *Endocrinology* 134: 2307-2316, 1994.
210. Zhou X, Kudo A, Kawakami H, and Hirano H: Immunohistochemical localization of androgen receptor in mouse testicular germ cells during fetal and postnatal development. *Anat Rec* 245: 509-518, 1996.
211. Bremner WJ, Millar MR, Sharpe RM, and Saunders PT: Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. *Endocrinology* 135: 1227-1234, 1994.
212. Iwamura M, Abrahamsson PA, Benning CM, Cockett AT, di, and Sant'Agnese PA: Androgen receptor immunostaining and its tissue distribution in formalin-fixed, paraffin-embedded sections after microwave treatment. *J Histochem Cytochem* 42 : 783-788, 1994.
213. Ruizeveld dWJ, Trapman J, Vermey, Mulder E, Zegers ND, and Van der Kwast TH: Androgen receptor expression in human tissues: an immunohistochemical study. *J Histochem Cytochem* 39: 927-936, 1991.
214. Sar M, Lubahn DB, French FS, and Wilson EM: Immunohistochemical localization of the androgen receptor in rat and human tissues. *Endocrinology* 127: 3180-3186, 1990.
215. Inkster S, Yue W, and Brodie A: Human testicular aromatase: immunocytochemical and biochemical studies. *J Clin Endocrinol Metab* 80: 1941-1947, 1995.
216. Levallet J, Bilinska B, Mittre H, Genissel C, Fresnel J, and Carreau S: Expression and immunolocalization of functional cytochrome P450 aromatase in mature rat testicular cells. *Biol Reprod* 58: 919-926, 1998.
217. Nitta H, Bunick D, Hess RA, Janulis L, Newton SC, Millette CF, Osawa Y, Shizuta Y, Toda K, and Bahr JM: Germ cells of the mouse testis express P450 aromatase. *Endocrinology* 132: 1396-1401, 1993.
218. Brinkmann AO: Molecular basis of androgen insensitivity. *Mol Cell Endocrinol* 179: 105-109, 2001.
219. Muller J: Morphometry and histology of gonads from twelve children and adolescents with the androgen insensitivity (testicular feminization) syndrome. *J Clin Endocrinol Metab* 59: 785-789, 1984.
220. Rutgers JL and Scully RE: The androgen insensitivity syndrome (testicular feminization): a clinicopathologic study of 43 cases. *Int J Gynecol Pathol* 10: 126-144, 1991.
221. Cottin V, Van Linden A, and Riches DW: Phosphorylation of tumor necrosis factor receptor CD120a (p55) by p42(mapk/erk2) induces changes in its subcellular localization. *J Biol Chem* 274: 32975-32987, 1999.
222. Gaeta ML, Johnson DR, Kluger MS, and Pober JS: The death domain of tumor necrosis factor receptor 1 is necessary but not sufficient for Golgi retention of the receptor and mediates receptor desensitization. *Lab Invest* 80: 1185-1194, 2000.
223. Jones SJ, Ledgerwood EC, Prins JB, Galbraith J, Johnson DR, Pober JS, and Bradley JR: TNF recruits TRADD to the plasma membrane but not the trans-Golgi network, the principal subcellular location of TNF-R1. *J Immunol* 162: 1042-1048, 1999.
224. Cottin V, Doan JE, and Riches DW: Restricted localization of the TNF receptor CD120a to lipid rafts: a novel role for the death domain. *J Immunol* 168 : 4095-4102, 2002.
225. Cottin V, Van Linden AA, and Riches DW: Phosphorylation of the tumor necrosis factor receptor CD120a (p55) recruits Bcl-2 and protects against apoptosis. *J Biol Chem* 276: 17252-17260, 2001.

226. Sata M and Walsh K: TNFα regulation of Fas ligand expression on the vascular endothelium modulates leukocyte extravasation. *Nat Med* 4: 415-420, 1998.
227. Li Z and Nabel GJ: A new member of the IκB protein family, IκBε, inhibits RelA (p65)-mediated NF-κB transcription. *Mol Cell Biol* 17: 6184-6190, 1997.
228. Thompson JE, Phillips RJ, Erdjument-Bromage H, Tempst P, and Ghosh S: I κBβ regulates the persistent response in a biphasic activation of NF-κB. *Cell* 80: 573-582, 1995.
229. Giwercman A and Skakkebaek NE: The effect of salicylazosulphapyridine (sulphasalazine) on male fertility. A review. *Int J Androl* 9: 38-52, 1986.
230. Levi AJ, Fisher AM, Hughes L, and Hendry WF: Male infertility due to sulphasalazine. *Lancet* 2: 276-278, 1979.
231. Toovey S, Hudson E, Hendry WF, and Levi AJ: Sulphasalazine and male infertility: reversibility and possible mechanism. *Gut* 22: 445-451, 1981.
232. Toth A: Reversible toxic effect of salicylazosulfapyridine on semen quality. *Fertil Steril* 31: 538-540, 1979.
233. Traub AI, Thompson W, and Carville J: Male infertility due to sulphasalazine. *Lancet* 2: 639-640, 1979.